

How to respond to Mr Andropov

President Reagan has listened to Mr Andropov (not before time) and is sending his vice-president to Europe. What follows is a check-list for Mr George Bush.

THE Reagan Administration seems finally to have heard what Mr Yuri Andropov is saying about opportunities for agreements on arms control: last week's remarkable declaration of the Warsaw Pact members in Prague can have given it very little choice. Whether it is an adequate response to send Mr George Bush, the Vice-President, on a fact-finding tour of European capitals is another matter; vice-presidents are usually listened to more attentively abroad than they are in Washington. But in case Mr Bush needs help of a kind that the State Department may not provide (and *Nature* makes no apology for its continuing interest in this corner of the political woods to which technology has a unique contribution to make), what follows is a simple guide to what Mr Andropov has been saying.

● **Who is for peace?** Everybody. So much can be told by comparing last week's long (and sometimes repetitive) declaration from Prague with the much more laconic declaration on arms control put out after the meeting of the North Atlantic Treaty Organization (NATO) in Bonn on 10 June last year, with President Reagan in attendance. Both documents emphasize the dangers in the accumulation of military arms in Europe and urge the need for "stable" (NATO) or "equitable" (Warsaw Pact) treaties to limit these at lower levels. Over the past few months, Western governments, singly or collectively, have been responsible for almost as many proposals for measures of arms control as are to be found in the Prague Declaration. President Reagan himself has been responsible for two important proposals — the "zero option" on intermediate nuclear missiles and the suggested reduction of 50 per cent in strategic missiles (compared with Mr Andropov's 25 per cent). The chief difference is that the Warsaw Pact countries keep saying that they are for agreement even when they are only half serious, but that NATO members (perhaps hamstrung by domestic diffidence in the United States) fail to say as much and thus lose the initiative to the East and to those legitimate domestic pressure groups campaigning against nuclear weapons.

● **What can be done quickly?** The simplest course would be to ratify two forgotten treaties — the Threshold Test-Ban Treaty (1974), which would require the Soviet Union and the United States to provide each other with seismic data invaluable for the continued monitoring of underground explosions, and that regulating peaceful nuclear explosions (1976), which for the first time admits of mutual inspection of the sites of nuclear explosions. It is pointless that these treaties should have been signed, and that the two parties should behave as if they were binding, while the positive technical benefits are denied. On the other hand, attempts at the ratification of Salt II would at this stage be counterproductive.

● **What comes after?** The Prague declaration contains at least 21 specific proposals for East-West agreements including several on which encouraging progress has recently been made. One of the best bets is for a treaty to prohibit the use of chemical weapons, being negotiated at the Geneva Committee on Disarmament. The comprehensive test-ban treaty (also now with the Committee on Disarmament since trilateral negotiations were abandoned in November 1980) includes valuable provisions on international inspection but is probably, at least for the US Senate, dependent on an agreement on strategic arms (now called START). The urgent need for 1983, however, is an agreement on intermediate-

range weapons in Europe, if only because the cruise and Pershing II missiles are due to be installed by the end of the year. The Prague declaration is mistaken in asserting that the West has imposed an "artificial deadline" on these bilateral negotiations, giving itself immunity from the effects of procrastination — no West European government would agree. But the British and French nuclear weapons will have sooner or later to be counted either as intermediate or strategic weapons, one reason why both sets of bilateral negotiations at Geneva must soon be merged.

● **What can wait, at least temporarily?** The Prague declaration is confusing in its completeness. Nuclear-free zones in Northern Europe and in the Balkans, desirable (but tricky) though they would no doubt be, are by precedent and principle matters for the states concerned. The proposed non-aggression treaty is in much the same case: but the West will sooner or later have to explain why its pragmatic way of conducting international relations persuades it to behave as if such treaties were not worth much.

● **What are the unexpected opportunities?** The Prague declaration contains two elements that Mr Bush should recognize as important — a ringing declaration that the reduction of conventional forces in Central Europe is also important, and a statement that a concerted policy on the supply of nuclear equipment and materials would be of powerful assistance in the restraint of the spread of nuclear weapons. The first proposal anticipates the intention of West European governments, the British chief among them, to revivify the MBFR (for mutually balanced force reduction) talks in Vienna, the longest running diplomatic show on earth, by presenting it with a draft treaty; the second is a splendid chance to make explicit the tacit understanding between the nuclear powers that they will keep their club as small as possible. There is also, on the strictly political plane, much to be said for the notion of a meeting between the governments of NATO and the Warsaw Pact.

● **What about the politics?** The Prague declaration is eloquent about the need that the Madrid conference on the Helsinki agreements, soon to reconvene, should do something substantial to strengthen the exchange of information, military and otherwise, helping to build confidence of one side in the other: that is an opportunity. The Prague and Bonn documents differ, however, on what they say about third parties — the Warsaw Pact insists that Western states should not assume the right to interfere in the affairs of third parties, the Bonn declaration that human rights become an issue wherever they are subverted by outsiders (Afghanistan, Poland). In reality, however, the disagreement is not as sharp as Mr Bush may think, and he should report in those terms when he gets back to Washington. He should also then describe in some detail the confusion that persists in Western Europe about his superior's wish to make the export of high technology to the Soviet Union impossible. The Prague declaration includes a long passage extolling the virtues of economic collaboration and social intercourse in Central Europe (incidentally offering to disband the Warsaw Pact if only NATO would agree to go out of business, which is a potentially poisoned chalice). The terms in which this is done would strike a responsive chord among those in Western Europe able to read them. Mr Bush will be hard-pressed, while making his rounds, to live up to that. He will have no choice but to do his best.



Deciding nuclear policy by argument

The next step in the British nuclear industry hangs on a public inquiry begun this week. The reasons why this is the wrong way to decide matters of public policy deserve to be more widely understood.

WHILE the post-Christmas pantomime season (men in drag, women dressed as boys) continues to fill the provincial theatres, the UK Department of Energy has provided a comparable event to keep the small village of Snape in Suffolk busy until the Aldeburgh music festival comes around again in the summer. For the public inquiry into the plan to build the first Britain pressurized water reactor (see page 102), which opened this week, will be pantomimic not merely in the sense that it is well rehearsed but also because the chief participants in this long drawn out affair will be required to dissemble. The Central Electricity Generating Board, which wants to build the reactor and eventually to operate it, has a reasonably straightforward objective — to make pressurized water reactors the chief means of generating nuclear energy in the decades immediately ahead; it will find itself required to go along with the assumption underlying the public inquiry that, in spite of the technical assessments of the safety of the proposed reactor which have been carried out in the past several years, others than those directly concerned will have the final say. And many of the objectors at the inquiry, who will no doubt concentrate their arguments on the weakest points in the generating board's case — the stagnation of electricity demand and the increased cost of nuclear reactors — will know that their case will be undermined if they openly declare their true beliefs that nuclear energy is an abomination.

This is the essence of the case that the public inquiry is a waste of time (see *Nature* 23/30 December 1982, p.672). That assertion is, of course, contentious, as readers' letters yet to be published will demonstrate, but the underlying issues are nevertheless crucial to the proper assessment of new technological projects in democratic countries such as Britain. The British tradition that there should be public inquiries into projects likely to cause an environmental nuisance was formally enshrined in the Town and Country Planning Act of 1947, amended in 1971, from which, however, agriculture and national defence are exempted, as are other publicly owned enterprises. The procedures that bring nationalized industries to public inquiries are different, stemming from the enabling legislation that allows the industry to exist and mandatory if a planning authority should object. In recent decades, ministers with contentious public projects on their hands have sensibly taken the view that public inquiries serve a useful purpose, not least because they give local populations an opportunity to question those responsible about the safety and seemliness of what is proposed.

Costs

The Sizewell inquiry, however, is different in its character. Deliberately, the Department of Energy has decided that the inquiry should be broad in scope, designed to deal with questions of whether pressurized water reactors are safe and even necessary. One of the intricate issues certain to be raised during the proceedings will be that of whether the Central Electricity Generating Board is on sure ground in its calculation of the costs of producing electricity from various fuels, an issue on which it was criticized by the Monopolies Commission a year ago. In the trade, the inquiry is called "generic" — and the benefits, both for the Department of Energy and the generating board, of a favourable recommendation would be that later plans to build pressurized water reactors might be decided on the nod. While that calculation is probably correct, it leaves out of account the damage that will be done to the quite separate arrangements that exist for making publicly acceptable decisions on such questions — and the high cost of the delay.

The duplication by the inquiry of the parallel arrangements for regulating the use made by the generating board of nuclear reactors is potentially the most damaging. As elsewhere, Britain now has an elaborate system whereby nuclear plants and the

operations of the electricity industry in general are supervised by constitutionally independent organizations. Thus licences for the construction and operation of nuclear power stations are issued by the Department of Energy only on the recommendation of the Nuclear Installations Inspectorate, which is maintained by a quite separate organization. The nuclear inspectorate has every incentive to be independent, and in particular to demonstrate its independence from the interests of those who wish either to build or to operate nuclear plants. Its opinion on the safety of the generating board's design, due to be heard towards the end of March, will obviously be important. If that opinion should be negative — at some stage, the inspectors will be asked "So would you recommend that a licence should be issued?" — the question of what the inquiry is for will be raised in the starkest form. If, on the other hand, the opinion is positive but the inspector, Sir Frank Layfield, disagrees, the question will be what the inspectorate itself is for.

Other issues certain to be raised in the months ahead are broader, the place of nuclear power in the British energy economy chief among them. Constitutionally, because British nuclear plants produce only electricity, public utilities such as the generating board have the formal say on what should be done. But the general operations of public utilities are subject to the approval of the Department of Energy (which has historically sought to encourage the use of coal), which can also give directions about capital investment. In the event, the department does not operate in a vacuum, but among other things is subject to a constant stream of advice from influential sources such as the select committees of the House of Commons. While the present government differs from most of its predecessors in not having an energy policy in the sense of not having published a prediction of how much energy will be won from different kinds of fuels in the years ahead, it is unthinkable that it could be diverted from its declared belief in keeping open all options by what is said at Sizewell. But if such a case can be miraculously established, will not the question arise of what parliament is for?

The issue of the cost of nuclear energy is even more tortuous in its constitutional implications. What matters is not the cost of nuclear electricity but that of electricity from whatever mixture of sources the generating boards have at their command. There are circumstances in which the addition of a relatively costly generating plant to a network may be the most economical course, if for example it can operate at more or less full load for most of the time. There is also something in the argument that it may be worth paying something in the way of capital cost for experience with a new kind of machine. There is little doubt that the generating boards (like other British nationalized industries) have in the past been too cavalier about the right to fix the prices charged for their products. Now, fortunately, the British government seems to have woken up to the importance of nationalized industries' prices, and is determined to constrain them. It is, however, hard to see how the Layfield inquiry could reject outright the generating board's economic case without calling into question the continued need for the Department of Energy. And if the inquiry is as daring, will those now planning to put objections to the inquiry then level similar complaints at the generating board's flirtation with windmills?

The absurdity of the Sizewell inquiry thus stems from the way in which broad questions of national and administrative policy are confused with the question of whether people living near the proposed reactor site will be exposed unduly to hazards, or whether an admittedly splendid stretch of coast is being needlessly developed. But are not open government and public accountability desirable objectives? Of course. The snag is that the inquiry now under way is a theatrical diversion from both objectives.

Cattle breeding

Techniques for sexing embryos now possible

Washington

CATTLE breeding for dairy and beef production could be revolutionized by a process for identifying the sex of 6-day-old calf embryos announced by Genetic Engineering Inc. of Denver, Colorado.

The company had announced the first successful use of its sex-identification process, when two female calves "Alpha" and "Beta", whose gender has been identified when they were embryos, were born naturally in December. Several more calves identified as females are due to be born in January and February. The company has begun identifying embryos and freezing them for shipment to customers.

Cattle are bred selectively for either dairy or beef. Females are needed almost exclusively for dairy herds, males almost exclusively for beef herds because they grow faster and have more meat.

Today, US cattle farmers routinely remove week-old embryos from their breeding animals so that they can conceive as often as possible. The embryos are then implanted non-surgically in surrogate mothers, which give birth naturally nine months later. Hitherto, the sex of a calf fetus could not be known until birth, so breeders had to feed the surrogate for nine months before knowing whether her offspring would be, in the case of dairy cattle, female and worth \$2,500 or male and worth \$50 as veal meat. Genetic

Male determinant

THE HY antigen used in Genetic Engineering's sexing test is a male determinant produced by the genes of the histocompatibility complex. Attempts to produce specific antibodies against the antigen have, however, mostly been frustrated. The British company International Embryos Limited (which says that its agreement with Genetic Engineering gives it the right to market sexed frozen embryos more widely than in South Africa) says it has been told that the sexing technique is 93 per cent accurate, suggesting good laboratory evidence that the monoclonal antibody developed by Wagner and Hoppe has high specificity.

The economics of the technique will, however, be determined not merely by the accuracy of the technique but also by the unknown degree to which embryos are damaged, or found unusable, in the laboratory. Cows used as a source of embryos are usually superovulated in advance, and their commercial value as sources of breeding material is determined by the number of viable embryos produced at each ovulation and insemination.

Engineering's sex-identification process would enable embryos only of the desired sex to be implanted in surrogates.

Genetic Engineering has filed a patent application for the new process, which Charles Srebnik, chairman of the company's board, would only describe as using monoclonal HY antibody and not removing any cells from the week-old embryo. The process was developed by Thomas E. Wagner of Ohio University, who is the chairman of the company's scientific advisory board and a stockholder in the company. The work was done at the company's laboratory in Denver, although the company does fund about \$200,000 worth of research at Ohio University Medical School.

The company expects an overseas market for its sex-typed embryos, especially in the Soviet Union, China and the Third World. Dairy cattle in developing countries produce 3,000 pounds of milk per year — whereas the selectively bred US dairy cow produces 16,000 pounds of milk a year. The intention is to sell frozen embryos abroad where they can be implanted in surrogate mothers of other breeds of cattle. The surrogate would then transfer her immunities to the fetus, enhancing its chances of adapting to the local environment after it is born.

The company has three joint venture arrangements: with Tower Tech International to sell embryos to Eastern Bloc countries, with the British company International Embryos Ltd to market in South Africa and with an Italian company for marketing in Italy and North Africa.

Despite its name, the company primarily does standard embryo transfer work for cattle breeders. In 1980, however, its name helped the company to raise \$400,000 through a limited offering and, in January 1981, to net \$3.3 million through a public stock offering of shares selling for \$5. For most of 1982 the stock hovered at between \$3½ and \$4½ per share, but after the announcement of the new sex-identification process, the share price rose, closing at \$6¾ on 31 December.

Company-sponsored research, however, include does gene-splicing. The work on the bovine growth hormone by Wagner at Ohio and Peter Hoppe at Jackson Laboratory, published in 1981, was sponsored under standard arrangements in which the company has first rights to any patentable product or process. Genetic Engineering had filed a patent for the bovine growth hormone process before Wagner and Hoppe published it.

Deborah Shapley

Research fraud

False data confessed

Washington

A FORMER researcher at Mt Sinai Medical School in New York City has admitted fabricating data that appeared in two scientific papers, a patent application that was granted, and a National Institute of Health (NIH) grant application and progress report.

The researcher, Dr Joseph Cort, was a part-time faculty member at Mt Sinai from 1976 to 1980. His research involved the synthesis of analogues of vasopressin and luteinizing hormone releasing hormone (LHRH).

Dr Cort's admission was made public late last month after Mt Sinai officials completed a 10-month investigation that began when Vega Biochemicals of Tucson, at the time Dr Cort's employer, informed Mt Sinai that Dr Cort had admitted fabricating some of his research. Cort subsequently admitted to a Mt Sinai fact-finding committee that he had not in fact synthesized one of five vasopressin analogues on which he reported results while at Mt Sinai. In examining laboratory records, the committee was unable to find evidence that a second vasopressin analogue had been synthesized either.

Laboratory records also showed discrepancies in a patent Cort received for the vasopressin analogues. Vasopressin is a substance that increases the blood level of clotting factor VIII, which is absent in haemophiliacs. Vasopressin has not been used to treat haemophiliacs, however, because among its side effects it is anti-diuretic and raises blood pressure. The fact-finding committee reported that Cort's claims for reduced side-effects of the analogues were exaggerated in the patent application.

A second patent application, now pending, refers to two LHRH antagonists that Cort claimed to have synthesized; the committee could find no evidence to support this in laboratory records. These compounds were claimed to have a potential as birth-control drugs.

Cort's research at Mt Sinai was principally supported by a contract from Vega, and according to Mt Sinai "no significant use of public funds was involved". Cort did, however, receive a small amount of support under a general NIH grant to the chairman of Cort's department.

Mt Sinai officials are informing the Patent Office and the journals and co-authors of the misrepresentations. The papers that included the fabricated data were published in *International Journal of Peptide and Protein Research* and *Advances in Physiological Sciences*.

Stephen Budiansky

Academic interest conflicts

Laxity of Californian practice

Washington

THE California Fair Political Practices Commission, which last March instituted a new financial disclosure policy for University of California faculty, has ordered an investigation of the university's enforcement of the rules. The action came after a six-month review of disclosure statements filed by faculty members revealed that 53 professors had a financial interest in the companies sponsoring their research, but that in no case did university review panels disapprove the grants or impose restrictions.

The review panels, made up of faculty members at each of the state-run university's campuses, bear the chief responsibility for reviewing the disclosure statements on which faculty must report any financial interests in a non-governmental research sponsor. The panel then makes a recommendation to the university chancellor on whether the grant should be approved.

According to the commission staff, 25 of the cases involved "significant financial interests" by principal investigators that give rise to "potential conflicts of interest in conducting the research". This financial interest usually took the form of consulting arrangements with the sponsoring company, although seven professors reported having investments of at least \$1,000 in the sponsoring company and two reported investments of more than \$100,000.

The staff provided several examples of significant financial interests that had been reported:

- Professor Michael Lovett, University of California, Los Angeles (UCLA), received a contract from Cetus Corporation for research on serodiagnosis of syphilis. His disclosure statement reveals consulting income from Cetus of \$1,000–\$100,000. Although the review panel at UCLA approved the application, Lovett was given a strong warning to "take great care that (a conflict of interest) . . . situation does not develop" and to avoid in particular "any inappropriate use of University facilities".
- Professor David Golde, UCLA, renewed an unspecified "collaborative agreement" with Genetics Institute. Golde reported holding 6,000 shares of Genetics Institute stock, worth \$30,000, plus vesting rights to an additional 69,000 shares. Golde is also a consultant on the board of scientific advisers of the company.
- Professor Orville Chapman, UCLA, earned more than \$10,000 as a consultant to Mobil Chemical Corporation over the past year and received a grant for research on carbon chemistry from Mobil.

"We were struck by the fact that no research was disapproved and no conditions were attached to any research application", said Robert Leidigh of the

Fair Political Practices Commission's legal division. "I'm not in a position to say they were rubber-stamped, but we don't have the documentation to tell."

So far, the commission has preferred to delegate as much responsibility as possible to the university to avoid concern over violations of academic freedom. Before last March, university faculty were exempted entirely from the disclosure requirements that apply to other state employees. But prompted by growing concern over faculty ties with outside companies and in particular by the case of Professor Ray Valentine of the University of California, Davis (who gave up a position on a research project funded by Allied Chemical after it became known that Allied had agreed to buy a 20 per cent interest in Calgene, a company founded by Valentine), the commission voted to lift the exemption (see *Nature* 296, 6; 1982).

Leidigh said the commission would not take any direct action on the 53 cases already approved by the review panels. He said also that the commission's decision on modifying the review procedure would hinge on how the panels perform over the next six months.

According to Afton Crooks, the university's conflict of interest coordinator, there are already indications that that performance may show some differences from that of the first six months. She said that there are "three to five" cases pending before review panels that have raised more serious conflict of interest questions than the 53 already approved, and that the recommendations of the review panel in one case at least "would substantially change the conditions of the research project". Details of these cases will not be available until a final decision is made by the chancellors.

Proponents of a tougher disclosure policy have seized on the findings of the commission. Al Meyerhoff of the Natural Resources Defense Council (NRDC) said, "It's even worse than we anticipated. This is far and away the largest number of potential conflicts that have come forth from any state agency". NRDC, together with California Rural Legal Assistance, has maintained that faculty should be required to disclose their financial stake in any company that might benefit from their research, regardless of the sponsor. The groups are particularly concerned about government-sponsored research at the University of California at Davis on agricultural machinery.

Crooks countered that "nobody has any statistics to back up his conclusion that this is a high figure". She said she had in fact expected many more cases of financial interests considering the "low threshold" (\$1,000 investment or \$250 income) for reporting.

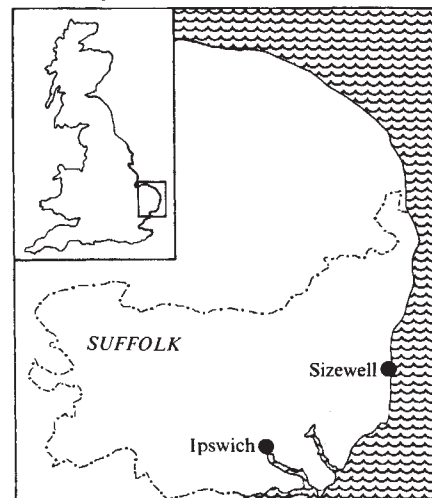
Stephen Budiansky

UK nuclear power

Sizewell for grabs

HIGH officials of the British Central Electricity Generating Board will spend roughly the next eight weeks in a building known as Snape Maltings, near the village of Snape in Suffolk. Eight weeks is the estimated time that will be needed by the board to put across to a public inquiry which opened on Monday this week the case for building a 1,100 MW pressurized water reactor, to be known as Sizewell B, on the coast a few miles north of the town of Aldeburgh.

Although the generating board first made public its wish to build Sizewell B at a parliamentary inquiry in 1972, the chance that it would be allowed to do so without formality was scotched in 1978 when Mr Tony Benn, then Minister of Energy in the British government, undertook that



neither the plan to build a pressurized water reactor nor that for building a commercial fast reactor (now put off indefinitely) would go ahead without a formal public inquiry.

The Thatcher government has appointed Sir Frank Layfield, a lawyer best known as chairman of the inquiry in the early 1970s into alternative methods of financing local government, as its inspector. He will receive technical assistance from Dr J. Vennart, director of the Medical Research Council's radiobiology unit, and from Mr Christopher Foster, a professor of economics at the London School of Economics. Arrangements have been made for the inquiry to move to London on 2 June and if necessary to return to Suffolk in September. The inspector's report to the Department of Energy is not expected before 1984; the minister is not bound to accept the recommendation.

The scope of the inquiry is deliberately broad, covering not merely the technical design of the reactor and questions of safety stemming therefrom but also questions of the need for nuclear energy, or indeed of new generating plant of any kind, when British demand for electricity is virtually stagnant.

Geothermal energy

Problems persist

TWO 2,000-metre boreholes for tapping geothermal heat in "hot dry rock" in Cornwall, England, by pumping water down one and out of the other, were "hydrofractured" just before Christmas to connect the two wells — but they remain stubbornly disconnected.

Cracks opened by the hydrofracturing (pressurizing the wells with water) have spread almost exactly along the plane of the wells, at right angles to the preferred direction (see figure). And on-line seismic mapping has indicated that the cracks have developed mostly downwards, rather than across the rock between the wells and have confined themselves to a narrow plane.

The result, according to Dr Tony Batchelor, director of the project, is that there is no direct, low-resistance flow path between the wells. Water flow between the two holes is thus only a fraction of that hoped for, and the thermal power extracted correspondingly reduced to 1 MW although it had been calculated that 30 MW might be available from the Cornish wells.

Even so, the Geothermal Energy Project of the Camborne School of Mines, which is responsible for the experiment, claimed that the £10-million exercise had been "80 per cent successful"; and Professor E.R. Oxburgh at the University of Cambridge described the results as "phenomenal".

The reason: Camborne techniques, which use an explosive charge to initiate cracks in the rock, followed by water pumping to open the natural joints, appear to have created a crack system more than 2 million m² in area and 100–200 m³ in volume. This is the scale of a major reservoir, and confirms the Camborne approach, says Oxburgh. The fact that the

cracks are parallel to the holes and mostly beneath them can be corrected with a third borehole, drilled to cross the cracks — at a cost approaching another £2 million.

The Camborne team expects to get its money as the first holes were considered experimental; and the UK Department of Energy, which paid £8.8 million towards the present holes (the balance having come from the European Commission), has "given permission" for a third hole.

But why do the existing holes seem to have been so "wrongly" placed, along the plane of maximum horizontal stress? It is partly bad luck: the holes were drilled to lie roughly between the planes of existing natural cracks, in the hope that either one or other would open when the holes were hydrofractured. In the event, the cracking opened along a zig-zag line in a plane determined by rock stresses, and the maximum stress happened to lie in the plane of the holes.

What is also clear, with hindsight, is that the stress directions were predictable. They were determined by tectonic forces in Europe and they correspond to stresses measured in a mine just ten miles from the drill site. However, what was unpredictable was the *ratio* between maximum and minimum stress. This has turned out to be high, and so determined the crack plane more effectively than the existing natural jointing structure.

Robert Walgate

Solidarity members to lose jobs

A MAJOR "reorganization" last month at the Nuclear Research Institute at Swierk, near Warsaw, has meant the "redundancy" of more than 60 former Solidarity activists, according to reports from the Solidarity underground in Warsaw.

Almost all those involved held senior scientific posts in the institute, many of the rank of full or associate professor. The proton synchrotron team, in particular, has been hard hit.

The chance of the redundant scientists finding appropriate employment in Poland seems remote. There is no unemployment benefit in Poland, and although the local underground committees of Solidarity still try to organize financial relief, their resources are stretched. Emigration would appear the best hope, but it is by no means certain that the authorities would grant visas to those affected.

The Swierk redundancies have raised fears that similar reorganizations may take place at other academic institutions where, formerly, Solidarity was particularly strong. Already there are signs of a coming shake-up in the Warsaw University Psychology Department, which was closed for three weeks in November following a protest strike.

Vera Rich

Medical research

Mandatory ethics recommendations

Canberra

THE National Health and Medical Research Council (NH and MRC) which administers a quarter of the total funds spent on medical research and development in Australia, has established a National Medical Research Ethics Committee which met for the first time on 13 December last year. The council will also write to all state and territory ministers of health recommending that it be made mandatory for all universities and hospitals in which medical research is undertaken to have institutional ethics committees and that the NH and MRC's recently revised statement and supplementary be brought to their attention.

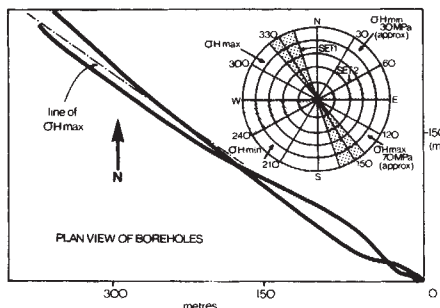
Its predecessor, the now disbanded Working Party on Medical Research, which recommended the setting up of the national body as well as revising the NH and MRC statement on human experimentation, was asked to look among other things at: (1) research on children, the mentally ill and those independent relationships; (2) therapeutic trials (3) *in vitro* fertilization and embryo transfer. Their conclusions were attached as supplementary notes to the NH and MRC statement.

At present all projects funded by NH

and MRC must comply fully with the NH and MRC statement and be cleared by institutional ethics committees. Although many other bodies administering grants for medical research also require prior approval by an institutional ethics committee, guidelines for approval may be arbitrary. Consequently the recommendation to states and territories will go some way towards creating the conditions for uniform requirements for all medical research projects and universal acceptance of NH and MRC guidelines in a country where health is a state government responsibility.

Under the chairmanship of Richard Lovell, professor of medicine at the University of Melbourne, the National Medical Research Ethics Committee will review ethical considerations of medical research and respond to questions referred to it by institutional ethics committees and government agencies. Its role in augmenting the authority of institutional ethics committees is a delicate one. Nevertheless, according to the working party report last August, it could, "by virtue of its well-informed and respected membership, act with such authority that uncooperative institutions and workers would see themselves as vulnerable".

Vimala Sarma



The compass rose indicates the directions of maximum and minimum horizontal stress (σH_{max} and H_{min}) in the rock, and the planes of the principal pre-existing joints in the formation (which are rotated slightly with respect to the present stress). Despite appearances, the holes nowhere intersect. The upper parts of the holes are cased, allowing water pumped down one to pass to the other through cracks in the hot granite. An explosive charge followed by water pumping at high pressure opened up a large surface area of joints zig-zag fashion almost parallel to the drill holes. The rock thus opened against the direction of minimum stress, rather than along natural joint planes.

East-West trade

Austria at odds with United States

Washington

THE Reagan Administration, which wants to control the flow of technology to the Soviet Union, has encountered problems in trying to persuade allied and neutral governments to do likewise.

Administration officials claim to have made progress in their negotiations to strengthen CoCom, the organization of NATO nations (minus Iceland and plus Japan) that approves technology exports to Eastern Bloc countries. The master list of items under CoCom's authority, they note, has been lengthened to include space launch vehicles, spacecraft and robotics.

But on other US initiatives, such as those to multiply CoCom's \$0.5 million annual budget fourfold and to add a military committee, "we have a long way to go" says one US official.

Some of the CoCom nations are arguing that they should not deprive themselves of trade with the Eastern Bloc if advanced neutral countries, such as Sweden, Switzerland, Finland and Austria, do not do likewise. So the US Administration has been engaged in delicate, secret negotiations with neutral countries in

The publication of Ikle's remarks brought a sharp retort from Austria's Chancellor Bruno Kreisky who promptly told *Kronen Zeitung* that Vienna was not a "transfer centre for smuggling western technology to the Soviet Union" and that for US officials to "prescribe" what Austria exports constituted "impermissible interference in Austrian domestic affairs".

The US State Department issued a statement on the day Ikle's remarks were published, apparently trying to smooth things over. It expressed confidence that "any problems in this area involving Austria can be resolved in a manner satisfactory to both sides", and said there was no question of imposing trade sanctions on Austria.

The US Administration, in its discussions with neutral governments, argues that they could assign their state security services to ensure that technology entering the country was not smuggled out or re-exported. It is also asking them to review exports not only to the Eastern Bloc but to other countries as well, and so not violate their neutrality. It is also trying to compensate for lax enforcement policies by the Carter Administration in the late 1970s.

Says one official: "In one case we allowed the sale of a major piece of technology involving network integration of radar systems to a country on the understanding it would not be retransferred. But it was retransferred in a complete violation of the understanding. We are now trying to create a climate in which we can feel confident about allowing our technology out in the first place."

Most other governments, including those of the CoCom countries, do not review the military implications of sales of civilian equipment and technology around the world. CoCom is supposed to provide this review by examining individual applications case by case for technologies on its list. Although founded in the 1950s to continue the post-war embargo, US officials say it has become a "rubber stamp" organization approving almost every application it receives.

The Administration has made five proposals for CoCom: updating the list, appointing a military committee, and increasing the staff and budget of CoCom's offices in Paris. It also seeks a stronger enforcement policy and harmonization of national laws on export controls.

But the CoCom governments are sceptical, one official said. "They are wondering whether the United States is really serious about this. **Devorah Shapley**

AND FURTHERMORE, NO MORE
McDONALD
FRANCHISES!



Europe and elsewhere to try to persuade them to limit their exports. Some of the neutral nations have been cooperative, but others object that such limitations would violate their neutrality.

Austria appears to have rebuffed the United States in December, after two high ranking and outspoken US defence officials, Fred C. Ikle, undersecretary of defence for policy, and Richard Perle, assistant secretary of defence for international security policy, told the Austrian press that the country should stop transferring technology to the Soviet Union and its allies. Ikle told *Die Presse* that Austria should erect barriers to technology transfer and that if the barriers "leak anywhere or collapse" then "we must draw the barriers closer and impose limitations". Perle made similar statements to *Kronen Zeitung* in November.

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French universities

Plans for a new reform

M. ALAIN SAVARY, French minister of education, has come down off the fence at last. After months of vacillation, he has produced a bill reforming the universities, *grandes écoles* and other institutions providing higher education in France. The bill is twice as long as its predecessor, Edgar Faure's solution to the student revolution of 1968, and yet it broadly maintains the *status quo*: a strong division between the *grandes écoles*, which train an elite corps of engineers and technocrats (and to which entry is highly competitive) and the universities.

This division, some had felt, weakened the universities (and hence, indirectly, research) and Savary was strongly advised to seek a more "comprehensive" system. The *grandes écoles*, which are strongly represented at high levels in government and industry, fought back. The latter have now clearly won.

Even so, the Savary bill is not entirely without innovation. The appointment of university lecturers and professors will be less centralized: instead of the universities proposing a short list, and a Paris-based committee making a final selection for each post, the procedure will be reversed: Paris will make a short list and the universities will choose, thus increasing university autonomy to a small degree.

Also, universities will be "contracted" to the state for a period of years, after

which a commission will assess the performance of the university and propose a new contract. And in each French *département*, a small "talking-shop" will be set up across *grandes écoles*, universities and other institutions to help coordinate teaching policy for the first year of higher education, to the eventual benefit of the student.

Another change is one of emphasis: the universities must groom their students for useful jobs — whereas Faure insisted that the universities were not "an employment bureau".

Meanwhile, however, several more contentious issues have been relegated to the form of "decrees": strictly, details of application of the law, but ones which can be determined by the council of ministers without the approval of parliament.

There are 45 of these extra-parliamentary decrees to come, none of them yet finished — so Savary's law may yet have a sting in the tail. Among them: an important reform of the higher degree system. According to present thinking in the ministry, the "troisième cycle" will be extended into a 3-4 year PhD (the present level is slightly lower), and the "these d'Etat" (a thesis going beyond PhD, and necessary for a university or research post) transformed into something like an assessment of previously published papers.

Robert Walgate

West Bank universities

Agreement without accord—as yet

Rehovot

SOME ten years ago Israeli and Palestinian academics began small, essentially clandestine meetings to discuss the possibility of working together. Now there are more frequent campus gatherings where Israelis and Palestinians alike can say in public what they had previously dared say only in private.

In the last days of 1982 the Weizmann Institute of Science in Rehovot held such a meeting for the first time. It was something of a milestone for this prestigious institution to act as host, and a lively and well mannered discussion took place in the institute's jam-packed Schmidt Auditorium.

Setting the tone of the meeting, the chairman Professor Joel Gat (a chemist) said that "the time has come for Weizmann Institute scientists long familiar with Princeton and Oxford to become acquainted with institutions like Bir Zeit, which are just around the corner". Seconding him, physicist Avraham Rinat attacked "extremists" — such as Rabbi Kahane on one side and George Habash on the other — who argue that the gulf between Arabs and Jews is unbridgeable. He added, "Dialogue between the two peoples is an act of reason, not of treason". Salim Tamari, Bir Zeit instructor in sociology, said that most Palestinians were now ready to come to terms with the existence of a state of Israel. He went on to claim that most of the staff and students at Bir Zeit, with the exception of the Moslem fundamentalists, favoured the establishment of a West Bank-Gaza Strip Palestinian state that would live in peace with Israel.

Khalil Mahshi, an educationalist and assistant to the dean of Bir Zeit's faculty of social science, listed the proliferating institutions of higher education on the West Bank and in the Gaza Strip. In Mahshi's view, too many academic centres are competing for a limited number of students and financial resources. What is required, he argued, is a greater emphasis on quality in education.

Bir Zeit assistant professor Rita Giacaman criticized the Israeli military authorities for having closed Bir Zeit from time to time and for harassing its students. She accused them of using "brutal and repressive" methods, and of trying to prevent the development of a Palestinian intelligentsia.

When pressed by members of the audience to explain how it was that West Bank universities had emerged only after 1967, under Israeli rule, she said that this was partly because, after 1967, those living on the West Bank had greater difficulty getting to outside universities, and that what had happened was a natural development that would have taken place

in any case.

But the Palestinian academics were also critical of the lack of freedom at universities in the surrounding countries. And when Mahshi was asked to compare what had happened at Bir Zeit with the situation at Amman University, he replied: "Maybe we have more freedom than is available to people in the Arab countries. But we are asking for the freedom that is available in Israel".

According to Dr Giacaman, formal exchange agreements between West Bank and Israeli institutions are not likely at this stage, although there is room for cooperation between individuals. She herself has been in close contact with public health experts at the Hebrew University for the past three years because "after a long process of dialogue, trust was established".

Since the Israeli authorities have apparently abandoned their attempt to force foreign lecturers at West Bank universities to pledge that they will not support the Palestinian Liberation Organization (PLO), this issue was hardly touched upon. And while the Bir Zeit people complained bitterly about the banning of books and periodicals in the West Bank that were freely available in Israel, none suggested that they were told what they could or could not teach.

Discussion at the Weizmann evening was so polite that some thorny issues — such as the reported PLO financing of West Bank universities and PLO terrorism — were virtually ignored.

At a subsequent gathering in a campus home, however, Tamari was faced with a difficult question from an institute

researcher: "What would happen to the Arab-populated sections of the Galilee if, as you suggest, Israel withdrew to her 1967 borders and a Palestinian State were established in the West Bank and Gaza Strip?" Taken aback, but still smiling, Tamari replied: "That would depend on how Galilee Arabs were treated. If they



Institutions on the West Bank and in the Gaza Strip: Bir Zeit (2,000 students); El Najah (2,500); University of Bethlehem (1,200); the Islamic College of Hebron (1,200) and the Islamic College of Gaza (1,300 students).

enjoyed real equality, they would undoubtedly wish to remain Israelis. If not," he said with a shrug of his shoulders, "who knows?"

Clearly there are many issues to be resolved before there can be a true rapprochement between Israelis and Palestinians. Yet many Israeli academics feel it is worth fostering a dialogue between the two groups. In the words of Weizmann Institute physicist Harry Lipkin, meetings such as this are "very much like the story of the talking horse . . . it's not what he said that was important; it's important that he talked at all".

Nechemia Meyers

Nature index of biotechnology stocks

1982 High	1982 Low	Company	Close previous month	Close 31 Dec.	Change
48*	16 1/8	A.B. Fortia (Sweden)	46 1/4	44 7/8	-1 3/8
8	2	Bio Logicals (Canada)	4 1/2	4 1/4	-1/4
9 1/8*	3 3/8	Bio-Response (USA)	6 1/2	7 5/8	1 1/8
14	7 3/4	Cetus (USA)	12 1/8	12 3/8	1/4
14 1/2*	6 1/8	Collaborative Research (USA)	13 1/8	11 7/8	-1 1/4
16 7/8*	5 3/4	Damon (USA)	12 5/8	16 1/8	3 1/2
26 1/2*	8 1/4	Enzo-Biochem (USA)	23 1/2	26 1/2*	3
28	6 5/8	Flow General (USA)	13 1/8	11 7/8	-1 1/4
49*	26	Genentech (USA)	40 1/4	41 3/4	1 1/2
13*	7	Genex (USA)	9 3/4	12 7/8	3 1/8
8 1/2*	2 1/4	Genetic Systems (USA)	6 3/4	8 1/2*	1 3/4
27 3/4*	9 5/8	Hybritech (USA)	25 3/4	22 1/4†	-3 1/2
15 1/4*	5	Molecular Genetics (USA)	14	14 3/4	3/4
27*	8	Monoclonal Antibodies (USA)	21	20 1/2	-1/2
52	34 7/8	Novo Industri A/S (Den.)	45	43	-1 3/4

The Nature Biotechnology Index for December 1982 stands at 157.5 compared with 157.4 for November. Base is 100 as of 25 June 1982. Previous indexes appeared in *Nature* 12 August, p.599; 9 September, p.101; 14 October, p.573; 11 November, p.101; and 9 December, p.475. Close of the month prices are the closing prices on the last Friday of each month. Where stocks are traded over the counter, the price quoted is the bid price. Where stocks are traded on the American Stock Exchange and New York Stock Exchange, the price quoted is the transaction price. Data from E.F. Hutton Inc. † Price adjusted to reflect issue of 1.2 million shares of additional stock.

* New high or low for calendar year.

Naming extraterrestrial life

SIR — The fields of extraterrestrial life, extrasolar planets, origin of life, theoretical biology, interstellar travel and communication, extraterrestrial intelligence and civilization, and SETI have no generally accepted collective nomenclature. Suggestions include astrobotany, astrobiology, bioastronomy, astropaleontology and astroarchaeology, cosmobiology (unfortunately linked to astrology), and biocosmology, bioastronautics, space biology, planetary biology, intellexobiology, and exosociology, each of which fails as a general term in taking as axiomatic that intelligence, technology and civilization require life or society, an arguable and imprudent assumption. The acronym SETI (Search for Extraterrestrial Intelligence) excludes biology and is inelegant.

Exobiology¹ is similarly flawed and is not universally accepted. Astronomy and Astrophysics Abstracts lists subject categories for extraterrestrial civilizations, intelligence and life, but not for exobiology. Physics Abstracts treats astrobiology and exobiology as minor keys to extraterrestrial life. NASA Thesaurus and International Aerospace Abstracts list all to avoid confusion. Many STAR Abstracts "exobiology" entries pertain to aerospace medicine, while the survival of Earth-life in space and life in the Solar System are also included under "extraterrestrial life". Further confusion is possible because "exobiology" has been coined by taxonomists to refer to future biology, in particular the prospects for a purely genotype-based biological taxonomic system². Xenobiology³ axiomatizes life, hence also fails. The most suitable word must be least limiting, suggesting the rootless forms exology and xenology.

Exo-/ex- (Greek or Latin, outside of, outer, outer part) words customarily describe a spatially displaced familiar, as in exogamy, exogenetic, exoplasm and exoskeleton. This does not reflect the necessity for the extraterrestrial entities under study not to be native to Earth. Xeno-/xen- (Greek, xenos, strange, a stranger, foreign) emphasizes the unfamiliar or foreign regardless of physical location, as in xenobiosis, xenogamy, xenogenetic and xenomorphic, hence is more appropriately applied to extraterrestrial studies. Further, ex-/exo- prefixes are already etymologically over-extended. My survey of 117 dictionaries and encyclopaedias published during 1959–79 reveals that exo- is 2.4 times more common than xeno-, and ex- occurs 97 times more often than xen-. No xen-/xeno-term is common, so xeno-neologisms are less likely to resemble existing words than exo- terms, which include some of the most common words in English⁴. The almost-

virgin xeno- prefix should be used to designate extraterrestrial entities (for example, xenobiont), concepts and subdisciplines (for example, xeno-biology, xenosociology), and exo- reserved for life outside of but native to Earth as in human space colonies.

A. G. W. Cameron (personal communication, 1979) claims "xenology" describes the study of xenon isotopic anomalies in rocks. Xenology in this sense was first coined by Reynolds⁵, but subsequent papers by him do not refer to the new term. Xenology also does not appear in the texts of papers citing Reynolds during 1969–79, and none of 17 papers on xenon meteoritic radiochronology selected randomly from this period mentions the new word in its text. (A better term is xenonology⁶.) Xenology was not used as a subject category by eight leading geological sciences abstracting services during 1963–79, nor in any physics, aerospace, NASA or geological sciences dictionary surveyed. Clearly Reynolds' neologism has negligible currency in the literature, whatever its informal status, so xeno- should not be considered pre-empted for extraterrestrial studies.

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What is life?

SIR — In the issue of 10 November 1982, (p.10), there is a letter from Dave Perry of Glasgow setting forth the view of the Catholic Church on the origin of life. I should like to point out that from the viewpoint of the legal profession, the beginning of life is implicit in the definition of the end of life which many jurisdictions accept: namely the cessation of electrical activity in the brain. If the end of such activity represents the end of life, then the beginning of such activity must represent the beginning of life.

There is, to the best of my knowledge, no reliable information available as to the time of onset of electrical activity in the brain, although I suspect that David Woolam of Cambridge has approached the problem.

It would seem that the subject should be investigated since the law appears to be thrusting a problem at science which has inadequate data to solve it. I would imagine that the religious community might also regard such research with some interest.

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UCCA admissions

SIR — The leading article in your issue of 25 November 1982 (p.302) contains the statement "... their (the universities') willingness to delegate the selection of the students whom they will teach to a computer centre in Cheltenham is a proof that they are only half serious". There is no such delegation and the statement is utterly false. One of the hallmarks of British university admissions is that they are conducted efficiently through a communications system (located in the office in Cheltenham) which leaves the decisions about admissions entirely in the hands of the admitting institutions.

The universities are entitled to expect a higher level of factual accuracy than this from Nature: to perpetuate such a grotesque and ill-founded inaccuracy destroys trust in the article as a whole.

HARRY KAY
(Chairman)

N.B. CHAPMAN
(Deputy Chairman)

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UCCA protests too much; the obvious point of the offending sentence is that too many universities make too little use of the responsibility left "in their hands". Editor Nature.

Naming problems

SIR — You published a letter (Nature **300**, 212; 1982) from an M.C. McKenna of the American Museum of Natural History commenting that the gender of nouns and pronouns in Chinese "remains confusing". McKenna states that the "Mr" Qi in the 16 September issue of Nature (p.200) should actually be "Mrs" Qi. May I ask why it should not be "Ms" if we are truly speaking about gender and not marital status? Furthermore, how are we to assess the gender of M.C. McKenna who gives as few clues to the gender of M.C. as we ordinarily have about Chinese personal names?

Finally, with the adoption of the pinyin system of transliteration by the Peoples' Republic in 1979, hyphens are no longer used in romanization. Thus, the only way for a foreigner unfamiliar with Chinese to discern which name is the family name and which is the personal is the rule of thumb that the personal name is usually (but not always) two syllables and the family name one. All in all, I find McKenna's comments out of date both in fact and in social niceties.

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Thermonuclear fusion 25 years on

Princeton's claim to have made its new tokamak function deserves acclaim. The meaning of this event is merely that the odds (against) viable fusion have lengthened — and that the decision time is closer.

THERE is, of course, no doubt that thermonuclear fusion can be made to work: such doubts as there are concern the feasibility of producing thermonuclear power economically. That is the aphorism against which must be assessed the significance of the report, elsewhere on this page, that the Princeton tokamak machine sprang into life last Christmas Eve.

In the year that happens to be the 25th anniversary of that in which Sir John (later Lord) Cockcroft announced that he was "99 per cent certain" that fusion had been demonstrated at the Harwell laboratory of the UK Atomic Energy Authority (see *Nature* 181, 217; 1958), it is natural that the people at Princeton should seek applause for having commissioned a machine that will probably (a few years from now) demonstrate that fusion can produce more energy than it consumes. Then, a few years later, the economist-minded engineers will come into their own and will be required to say whether expenditures perhaps ten times as large as at present each year will be justifiable: fusion will have moved from being a zero-odds gamble on an infinite pay-off to being a ten-to-one (against) chance that industrialized countries will be able to save a substantial fraction of, say, ten per cent of their gross domestic products.

Those who accuse governments of shortsightedness in the pursuit of research should think again; the motives of those that continue generously to support close on a hundred thermonuclear machines of various kinds and scales cannot be simply electoral.

Meanwhile, there is a case for admiring (in the Latin sense of "to wonder at") what has been accomplished. A quarter of a century ago, the neutrons emitted from Harwell's experimental machine turned out to be neutrons produced in predictable kinds of nuclear reactions; it is remarkable that people did not promptly give up and turn to solid-state physics or something even safer. With the passage of time and thus the accumulation of experience, the criteria for success in the chase towards the Holy Grail have become more clearly understood. Nobody now supposes that it will be possible to make an artificial sun that will sustain itself in being until its light-element fuel is exhausted; the best hope is to create a transient sun that will operate at higher temperatures than the sun that keeps us warm (ideally, at ten times the central temperature of 15 million K), do so for a few microseconds at a time and allow the unburned materials to be recycled.

The appropriate "figure of merit", as the engineers say, is the product of the average ion (i.e. nuclear) density and the confinement time, otherwise the Lawson

number. In all schemes for making thermonuclear fusion possible, the guiding principle has so far been that only magnetic fields are so immaterial as to be undamaged by the impacts of material particles bent on escape. The ZETA machine, called a "fast-pinch machine", made the ions of a plasma into what was nothing but the (single-turn) secondary of an electrical transformer,

with positive and negative ions (electrons) travelling in opposite directions, and producing its own magnetic field with a rapidly increasing radial gradient so as to compress the plasma in the middle.

Tokamaks are different. Given the knowledge that plasmas may be confined by patterns of magnetic force running axially around toruses, it was possible (but not easy) to predict that such a pattern of forces artificially created would provide a means of confining a hot plasma, whence the tokamak. The essential trick is to accelerate the particles of the plasma, the ions in one direction and the electrons in the other, in such a way as to increase their mutual temperature.

Most magnetic confinement machines in which hot plasma is confined by toroidal patterns of magnetic fields work in the same way. While the past quarter of a century has shown that, in devices of this kind, it is possible to predict what modes of instability are likely to occur and to take steps to keep them small, the magnitude of these effects and their interactions can be understood only by building a machine and seeing how it works. More recently, it has been recognized that turbulence as distinct from gross instability is important even when every step has been taken to prevent a nominally toroidal plasma turning into a corkscrew turned in upon itself.

Thus it has come to be recognized that fast-pinch machines are at one end of the spectrum (high turbulence), stellarators (like the original Princeton machine) at the other and tokamaks somewhere in between. For the time being, however, the need that there should be some kind of a machine that will make possible experimental study of thermonuclear fusion on a substantial scale is sufficient to justify the building of compromise tokamaks — at least a dozen of various sizes in Europe alone.

In the end, however, it could well turn out that some quite different machine — a "mirror" machine in which magnetic confinement requires that particles should bounce from one end of a magnetic "bottle" to another, or something involving a giant laser, for example, may offer the best chance of thermonuclear power. One recent trouble is whether magnetic fields capable of containing plasmas at a pressure sufficient to yield usable amounts of thermonuclear power can be generated by magnetic coils with reasonable mechanical strength. For the time being, then, the question is not whether the machine at Princeton, or which other machine, will function economically but whether any will. Only time, and quite a lot of it, will give the answer.

US tokamak starts up

Washington

SCIENTISTS at Princeton's Plasma Physics Laboratory have successfully started up the first of a new generation of tokamak fusion devices. The \$314 million Tokamak Fusion Test Reactor (TFTR) is expected to attain scientific break-even — the point at which as much energy is produced by the fusion reaction as is applied to the plasma — by 1986.

The initial test of the machine, on Christmas Eve, produced a hydrogen plasma that lasted only 1/20th of a second. Its temperature of 100,000 °C was two orders of magnitude below that needed to attain break-even. The director of the laboratory, Dr Harold Furth, warned that some press reports of the event may have exaggerated its significance. "It's not a scientific event", he said, "but we were absolutely astonished that it worked on the first shot." The enthusiastic reporting was probably not dampened by the timing of the event, which fell on the slowest news week of the year, nor by Dr Furth's earlier statement, "It's like Columbus finding the New World".

TFTR is, however, the first tokamak machine that will be able to produce a plasma substantial enough to attain scientific break-even. "At the present rate, TFTR should reach reactor parameters in 1986", Furth said.

Initial testing will be done with hydrogen and deuterium plasmas. By the end of the year, the Princeton team expects to have ready the powerful heating method that uses energetic neutral beams to raise the plasma to the required temperature for fusion to take place.

The final step towards the break-even point should come in 1986, when deuterium/tritium plasma — which undergoes fusion at a lower temperature than straight deuterium, but also produces radiation in the form of alpha particles — is introduced into the machine.

TFTR's closest competitor will be the Joint European Torus (JET) at Culham in the United Kingdom, which is due to come on line this summer. Japan's JT-60 and the Soviet Union's T-15 are due to be completed in 1985. Stephen Budiansky

Gene therapy

Controlling the fetal globin switch in man

from Stuart H. Orkin

A DARING and provocative attempt to manipulate globin gene expression in a patient with severe β -thalassaemia has just been reported in the *New England Journal of Medicine* (9 December, p.1469) by Ley and colleagues¹. The nucleoside analogue 5-azacytidine (5-azaC) — known to inhibit DNA methylation *in vivo* — was administered in an attempt to switch back on the fetal haemoglobin genes that are repressed in development.

The transition from fetal to adult life in man (and other vertebrates) is marked in erythroid cells by the repression of fetal γ -globin genes and the reciprocal activation of adult β -globin genes². It is at this stage that the disastrous effects of mutations of the β -globin genes, seen notably in β -thalassaemia and sickle cell anaemia, first become apparent. Reversal of the fetal to adult haemoglobin switch could allow treatment of many affected individuals. Furthermore, an understanding of the mechanisms underlying the switch could provide general insights into other developmental switches in gene expression.

The attempt to derepress human fetal globin genes reported by Ley and colleagues and an earlier attempt with baboons take as their starting point the accumulating evidence for an inverse correlation between gene activity and the extent of DNA methylation (see ref. 3 for review). Although exceptions have been described, the association suggests an important role for DNA methylation in the regulation of gene expression during development. Consistent with this hypothesis, deliberate demethylation of DNA following treatment with 5-azaC has been associated with induction of expression of several genes in cultured cells^{3,4}. In fetal erythroid cells, the γ -globin genes are relatively hypomethylated, whereas they are more highly methylated in adult bone marrow cells where γ -globin synthesis is minimal⁵.

Given these findings, DeSimone and his colleagues⁶ asked might not treatment with 5-azaC activate repressed γ -globin genes in adult baboons, whose haemoglobins and globin genes are very similar to those of man? Although many of us considered such an experiment naive and unlikely to yield positive results, DeSimone *et al.* witnessed dramatic transient increases in fetal haemoglobin production in treated animals. Since bleeding and hypoxia also readily induce a reverse fetal switch in the baboon, whereas their effects are much less striking in man, one might argue that this response to 5-azaC treatment reflects merely a peculiarity of the species rather

than a more general biological phenomenon.

The new studies in man have quickly dismissed this provincial view. In a carefully designed and executed experiment, Ley and associates at the NIH¹ administered 5-azaC to an adult β -thalassaemic patient for whom conventional therapy had little more to offer. As is characteristic of patients with β -thalassaemia, β -globin synthesis was severely impaired before treatment. Stimulation of γ -globin synthesis, if it were possible, would replace missing β -chains with γ -chains, reduce the globin chain imbalance and modify the disease. After seven days' continuous infusion of 5-azaC Ley and his colleagues observed a substantial increase in γ -globin chain synthesis, reduced chain imbalance and extensive hypomethylation of DNA in the vicinity of the γ - and the embryonic ϵ -globin genes. Within two weeks a burst of reticulocytes (young red cells) appeared in the blood, followed by a rise in the haemoglobin content. No further blood transfusion was needed for a six week period following the one week infusion. Subsequent treatment of two other β -thalassaemics and two patients with sickle cell anaemia resulted in similar increases in fetal haemoglobin production and haemoglobin levels. Similar effects after repeated treatment of a single sickle cell patient have now also been observed with a lower dose of 5-azaC by Dover, Charache and colleagues at Johns Hopkins University⁷.

These clinical investigations raise many important questions and issues. First, how does 5-azaC produce the effect on haemoglobin synthesis? The experiments were founded on the simple premise that demethylation of γ -globin gene DNA in erythroid cells would lead to increased expression. Whether the principal effect of the drug is via this route is uncertain. For example, although demethylation of the ϵ -globin gene was as extensive as that of the γ -genes, only minimal expression of ϵ -RNA was observed in the patient treated by Ley *et al.* Alternatively, 5-azaC could act by perturbing the population of red cell precursors in the bone marrow. Since the drug is cell-cycle specific⁸, dividing progenitors, which are committed to the synthesis of adult haemoglobin rather than fetal haemoglobin, are most sensitive to its damaging effects. The entry of 'earlier' progenitors into cycle may well lead to the observed burst in fetal haemoglobin production. That erythropoiesis in man can draw upon these cells is evident from the increased fetal haemoglobin levels

found associated with chronic marrow stress and following bone marrow transplantation⁹. Of course, demethylation of DNA and disturbance of progenitors in the marrow may both be involved in producing the effect.

Second, what toxicities may be associated with attempts to manipulate globin gene expression in this manner? 5-azaC has been used for several years in the management of acute myelogenous leukaemia. Short-term side effects are largely known and were minor in the β -thalassaemic and sickle cell patients. Nevertheless, the long-term effects of this potent drug are virtually unknown. Potential carcinogenicity has not been fully assessed. Promiscuous demethylation of DNA is unlikely to be totally benign². We are left to speculate about the possible induction of expression of a host of genes (oncogenes, for example) whose effects might be catastrophic^{3,10}. Given these uncertainties, Ley and his colleagues rightly stress the need for careful studies of drug toxicity and optimal dosage scheduling. Further studies should be performed only in consenting adult patients for whom conventional medical management has failed and patients should be considered case-by-case under the scrutiny of institutional human investigation committees.

Third, will 5-azaC induce a quantitatively similar effect in all individuals? Probably not. The capacity of baboon and man to respond to various stimuli with increased fetal haemoglobin production appears to be under genetic control¹¹. The locus responsible for these differences is unknown. Classes of patients with genetically determined differences in responsiveness may exist. Furthermore, among patients with β -thalassaemia the defect in β -chain synthesis is heterogeneous and it is uncertain whether the improvement in chain imbalance described by Ley *et al.*¹ is typical of those with the most common varieties of the disease. If manipulation of globin production by drug treatment is to become a useful clinical tool such factors will have to be carefully considered.

Fourth, in view of all this, can we foresee a role for 5-azaC in clinical management of either β -thalassaemia or sickle cell anaemia in the near future? Certainly an increase in γ -globin synthesis could help individuals affected with β -thalassaemia by decreasing transfusion requirements and those with sickle cell anaemia by boosting fetal haemoglobin enough to prevent sickling.

The apparent success of the 5-azaC experiments now poses a difficult dilemma: should this potentially dangerous drug be used in attempts to treat these chronic disorders? And if so, under what circumstances? For many, the administration of this agent to man will be unacceptable under any circumstances until we know considerably more about potential toxicity. On the other hand,

many would gladly accept uncertain long-term effects in exchange for immediate benefits in the management of some patients with intractable sickle cell anaemia or end-stage β -thalassaemia. As an example, for severely iron-loaded β -thalassaemic patients, conventional therapy — that is continued transfusions — does indeed present an immediate danger. In the foreseeable future, though, treatment of children cannot be justified. Open discussion of these issues should be encouraged. In short, it is premature to believe that effective and safe treatment for these conditions has finally arrived. We must temper the understandable enthusiasm to treat with our ignorance regarding long-term consequences, particularly those associated with activation of other cellular genes.

Reservations concerning the ultimate usefulness of 5-azaC, however, do not detract from the remarkable nature of the biological phenomenon that has been described: administration of an exogenous agent can significantly influence the pattern of haemoglobin production in man. Understanding the precise mechanism by which 5-azaC exerts this effect is

critical. If it is principally via demethylation of DNA, this approach to controlling the fetal globin switch for clinical benefit may be doomed. It is unlikely that an agent capable of demethylating large stretches of the genome will be safe for chronic administration. On the other hand, if the effect is via perturbation of marrow progenitors, the chances of finding a suitable, relatively nontoxic agent will be more hopeful. □

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Alzheimer's disease

Untangling insoluble filaments

from Brian Anderton

SENILE DEMENTIA of the Alzheimer type (SDAT) is a serious medical and social problem. As much as 5 per cent of the population are affected at age 65, rising to 20 per cent at age 80. SDAT has a well defined pathology and two histological features of cerebral cortical neurones correlate with the severity of dementia — the number of neurofibrillary tangles and the number of senile plaques. Deficits in synthesis of certain neurotransmitters, the best documented being that of the cholinergic system, have recently been discovered and this has raised hopes of therapy by compensating for depressed acetylcholine levels. As yet, though, we are unable to relate any neurochemical defects to the classical histological abnormalities, let alone understand the pathogenesis of SDAT. To bridge this gap it would be of great help to understand the chemical nature of the neurofibrillary tangles. Some recent reports from Dennis Selkoe's laboratory at the McLean Hospital, Boston describe progress that may be a turning point in this very difficult area of investigation.

Like a number of others, Selkoe and his colleagues have been trying for some time to isolate neurofibrillary tangles or, rather, the paired helical filaments of which they are composed. The paired helical filaments (PHF) themselves contain pairs of 10 nm wide filaments which twist about each other to produce a helix with an 80 nm

pitch^{1,2}. Enormous aggregates of them make up the neurofibrillary tangles that fill the perikarya of many neurones in the cerebral cortex. PHF are also seen as smaller aggregates in the swollen degenerating neuronal processes found in the senile plaques — areas of localized abnormal processes often surrounding a core of amyloid and appearing round in histological sections.

What Selkoe and his group have now discovered is that PHF have remarkable chemical properties. They are insoluble in all the usual reagents capable of dissolving macromolecular assemblies, including Triton X-100, SDS, urea, guanidine hydrochloride, acid and alkali³. Although this makes the analysis of PHF difficult, it makes their isolation easy; most other biological molecules can be extracted in these harsh conditions, the PHF remaining insoluble and still in their helically twisted state. Selkoe and colleagues propose that the insolubility of PHF stems from the cross-linking of proteins by non-disulphide bonds and postulates the most likely candidate to be a γ -glutamyl- ϵ -lysine cross-link catalysed by transglutaminase, a calcium-activated enzyme.

In a second paper that has just been published, the same group has studied the cross-linking of brain neurofilaments catalysed by brain transglutaminase⁴. Neurofilaments are straight and 10 nm wide and thus of the right dimensions to

form PHF. There are several reports that antibodies to neurofilaments and other 10 nm intermediate filaments also stain neurofibrillary tangles although not all neurofilament antibodies have anti-tangle reactivity⁵⁻¹⁰.

Transglutaminase activity was thought most likely to be responsible for the insolubility of PHF since the γ -glutamyl- ϵ -lysine cross-link is the most common non-disulphide cross-link found in protein polymers. The physiological function of the cross-link is nevertheless not known except in a few cases, such as the last step of the blood clotting sequence (factor XIIIa is plasma transglutaminase) and in formation of skin epidermis by cross-linking of envelope protein in terminally differentiated keratinocytes^{11,12}.

The cross-linking of neurofilaments by transglutaminase is not, as Selkoe and colleagues acknowledge, proof that they form PHF. The *in vitro* cross-linked neurofilaments did not twist about each other and they were not the only proteins to be cross-linked. Also, the formal proof that transglutaminase is responsible for PHF insolubility will require isolation of the γ -glutamyl- ϵ -lysine isodipeptide from PHF. Nevertheless, the discovery that PHF are insoluble will spur on protein chemists to isolate PHF in homogeneous form. Once the reason for their insolubility is established, it should be possible to guess at what has gone wrong with the affected neurones. Should transglutaminase indeed be important, then a search for abnormal calcium metabolism would be justified.

Isolated PHF will also have to be examined immunochemically to see why tangles show some cross-reactivity with neurofilaments and also to finally nail down the normal cellular constituents that end up forming PHF and tangles in diseased neurones. The work from the McLean Hospital has provided a fresh stimulus to this research and, with luck, we may soon begin to link neurochemical changes with the long observed pathology. □

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Memory effects in ridge crests

has been produced north of the fracture zone. This could be achieved either by asymmetric spreading or by a ridge jump, both of them processes that are known to occur. The effect of asymmetric spreading on the magnetic anomalies in our example between points *a* and *b* would be to extend the wavelength of the magnetic anomaly, so that the troughs of the (say) negative anomaly between *a* and *b* should still lie on average mid-way between the lineations *a* and *b*. It is therefore quite valid to do as Schouten and Klitgord have done and map these peaks and troughs. However, in the area between anomalies 20 and 21 mapped in the North Atlantic at 45°N (ref. 4) identical geometry to that of anomalies *a* and *b* is found and south of the fracture zone an additional 'wobble' has been mapped in the magnetic anomalies. This indicates additional crust belonging to another magnetic polarity event which might have been juxtaposed here by a ridge-jump, that is, when the location of the ridge crest itself changed, thus destroying the expected pattern of magnetic lineations. If variable offsets over fracture zones are due to ridge jumps, it seems even more difficult to visualize a memory effect remaining constant relative to the ridge crest.

The Schouten and Klitgord model proposes a high degree of order in the structure of the sea floor which leads to the question of the scale of order in nature — or at least the scale of order in the Earth's crust. On the one hand plate tectonic theory predicts constancy on a regional scale but on the local scale many a well surveyed area has turned out to be anomalous. Our perpetual hunt for 'normal' sea floor has often proved elusive.

How real are the kinks in magnetic lineations? Far from giving a greater understanding of ocean crust magnetization, the Deep Sea Drilling Project has shown us how complicated and chaotic crustal magnetization is. We also know that the elongate spreading cell is a simplified generalization since the random discrete volcano-injection pattern was observed in the FAMOUS area⁵. On the scale of macro lineations, however, order exists and the magnetic dataset south-west of Bermuda and its interpretation is quite convincing.

The point in their paper about which I remain most sceptical is the stability of the spreading cell system. Intuitively I feel that once a fracture zone forms and its immediate crust cools, even if it later becomes a zero-offset fracture zone there will be some time delay before its crust can re-heat and form part of a spreading cell. It may well be that the time scale for occurrences of changes in stress is shorter than the 40–50 Myr time scale required for re-heating the crust. Thus should a change in stress occur within this period, the former fracture zone location is still a zone of weakness and is likely to be the location of a new-generation fracture zone, but if

no changes in stress occur the fracture zone location ought to heat up and become part of the spreading cell.

Schouten and Klitgord see the picture having more constancy than this and view the spreading cells in the ocean floor as stationary features separated by fracture zones along which anomalous crust is formed and that once formed these will remain anomalous and will always be the sites of offset or non-offset fracture zones. They see a high degree of order in the structure of the sea floor. Such optimism

deserves encouragement, I hope they are right! It would certainly make cruise planning easier. □

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Chromosome translocations

Still more about oncogenes

from Peter Newmark

1982, the year of the oncogene, went out with a flurry of papers on *c-myc*, the cellular oncogene whose activation in virally induced chicken lymphomas (*Nature* **290**, 475; 1981) fired much of the current research.

Five papers document evidence (some of it already publicized through meeting reports — see *Nature* **300**, 403; 1982) that links *c-myc* to both Burkitt's lymphoma and mouse plasmacytomas.

The link to Burkitt's lymphoma is made three times over in the last issue of the *Proceedings of the National Academy of Sciences* for 1982 (volume 79). Dalla-Favera *et al.* (p.7824), Taub *et al.* (p.7837) and Neel *et al.* (p.7842) independently locate the *c-myc* gene at band q24 of human chromosome 8, within the segment that is translocated from chromosome 8 to 14 or, less frequently, to chromosome 2 or 22 in Burkitt's lymphoma.

Taub *et al.* go on to show that in eight out of twelve Burkitt's lymphoma cell lines, one of the two allelic copies of *c-myc* has been rearranged and that in two of the cell lines the rearrangement is clearly the result of the translocation since it has placed *c-myc* in proximity with the immunoglobulin heavy-chain region on chromosome 14. Dalla-Favera *et al.* mention similar evidence in a note added in proof; the details are soon to be published.

The link between *c-myc* and mouse plasmacytomas is also made three times over. Both Taub *et al.* (op.cit) and Crews *et al.* (*Science* **218**, 1319; 1982) show there to be *c-myc* sequences in a non-immunoglobulin-related sequence attached to the immunoglobulin heavy-chain gene region of several mouse plasmacytomas. The heavy-chain genes are on chromosome 12 and evidence already exists (see *Nature* **300**, 477; 1982) to show that the non-immunoglobulin-related sequence attached to it comes from chromosome 15 by translocation. Obviously, the translocation takes *c-myc* from chromosome 15 and places it on chromosome 12 at the heavy-chain locus.

To be more precise *c-myc* finishes up in

the 'switch' region of the heavy chain locus according to both Taub *et al.* and Shen-Ong *et al.* (*Cell* **31**, 443; 1982). The latter provides several details of the fate of the translocated *c-myc* gene. First, the *c-myc* and the heavy-chain genes are found in opposite orientations, a conclusion confirmed by Crews *et al.* Second, in each of four plasmacytomas with a translocated *c-myc*, Shen-Ong *et al.* found a species of *myc* RNA transcript about 400 bases shorter than normal, the suspicion being that one exon had been displaced by translocation. Finally, the level of *myc* RNA is no greater than normal.

The question that is left open is whether the shift of *c-myc* from its normal chromosome to the immunoglobulin heavy-chain locus in both man and mouse is important for the induction of the lymphoma/plasmacytoma or is simply an irrelevant consequence of the translocation. The evidence of Shen-Ong *et al.* counters any suggestion that tumour induction is the result of increased transcription of *c-myc* in its new locus, but raises the possibility that an altered *c-myc* product is the problem. On the other hand, as several of the papers emphasize, in a sizeable minority of Burkitt's lymphoma lines and plasmacytoma mouse there is no evidence of any *c-myc* rearrangement.

Another note of caution is sounded by Crews *et al.* They have transformed NIH 3T3 cells with mouse plasmacytoma DNA and have shown that it is not the translocated *c-myc* sequence that is responsible for transformation, a conclusion that fits with Cooper and Neiman's observation that it was not the activated *c-myc* of virally induced chicken lymphomas that transformed NIH 3T3 cells (*Nature* **292**, 857; 1981) but another presumptive oncogene. Does this mean the NIH 3T3 assay is dead? Or that *c-myc* is irrelevant to the lymphoma? Or should one accept the somewhat glib explanation that a multistep process of oncogenesis has been observed. The answers should emerge in 1983. □

Peter Newmark is Deputy Editor of Nature.

Eukaryote genetics

Cloning chromosome ends

from T. Cavalier-Smith

CHROMOSOMES consist of single DNA molecules that may be either circular (in bacteria) or linear (in eukaryotes). It has long been clear that the ends of eukaryote chromosomes — the telomeres — differ markedly from artificial ends made by breaking a DNA molecule. Telomeres are very stable as free ends, whereas ends of molecules broken *in vivo* tend to stick together irreversibly, presumably covalently through the activity of repair enzymes. Furthermore, natural ends require a special mechanism to replicate the 5' ends of daughter molecules since DNA polymerases can polymerize nucleotides only onto a pre-existing primer¹. Direct molecular studies of telomeres have been hampered by the difficulty of distinguishing them from the accidentally broken ends that predominate in preparations of eukaryote DNA. Even conventional recombinant DNA techniques cannot overcome this problem for they use circular plasmids for cloning. However, Szostak and Blackburn² have now constructed a linear plasmid so as to be able to clone eukaryote telomeres.

They used a naturally occurring linear plasmid from the macronuclei of the protozoan *Tetrahymena* — the rDNA plasmid. They cut off its ends and spliced them onto the two ends of a linearized derivative of a circular yeast plasmid. The chimaeric linear plasmid could be taken up by yeast cells and accurately and stably replicated, showing that the yeast telomere replication mechanism can recognize *Tetrahymena* plasmid telomeres. This implies that the mechanism of telomere replication is highly conserved; and since *Tetrahymena* and yeast are in separate eukaryote kingdoms, all eukaryotes probably use the same basic mechanisms.

Szostak and Blackburn went on to use their new linear plasmid as a cloning vehicle

for yeast telomeres, by selecting for restriction fragments of yeast DNA that could replace one of the *Tetrahymena* telomeres. They isolated several such sequences and used one as a radioactively labelled probe to show that there were 30–40 copies per genome of DNA sequences able to hybridize with it. Since the yeast *Saccharomyces cerevisiae* has 34 telomeres this is consistent with their all being homologous, as previously suggested^{1,3}. Such homology was confirmed by restriction mapping the three independent telomeric sequences. Although the DNA sequence has not been determined, indirect labelling experiments using DNA polymerase strongly suggest that single-strand interruptions are present in an A + C-rich sequence near the termini, as in the *Tetrahymena* plasmid which has from 20 to 70 repeats of the sequence CCCCAG at each end.

Similar sequences (several repeats of CCCCAG) are present at the ends of the gene-sized DNA molecules found in the macronuclei of hypotrich ciliate protozoa such as *Oxytricha*. *Oxytricha* destroys over 90 per cent of its different DNA sequences during the formation of its macronuclei, leaving only about 25,000 coding sequences which replicate as gene-sized molecules. Prescott's laboratory⁴ has now cloned and radiolabelled a segment of the C₄A₄ repeat to study the distribution of these sequences in micronuclear DNA, from which the macronuclear DNA is produced during development. Even though such sequences are present at the ends of virtually all the macronuclear DNA, they are not present at corresponding places in cloned micronuclear DNA sequences shown by hybridization to be homologous to macronuclear molecules. This suggests that the ends of the macronuclear gene-

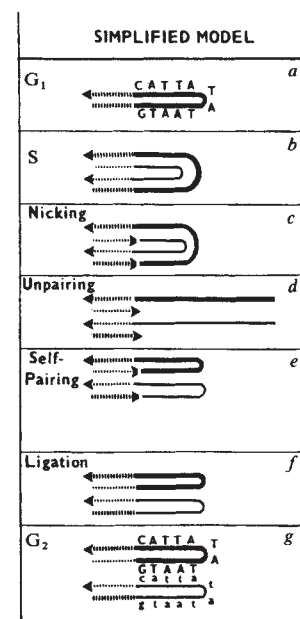


Fig. 2 Bateman's simplified version⁵ of my hairpin theory avoids the terminal gap-filling problem. When not being replicated, chromosome ends are hairpins covalently bonded throughout their length (a). When these are replicated (b) the sister chromatids are unable to separate until one strand in each is cut by an endonuclease in a specific manner (c), and the base pairs holding them together are broken (d). The single-stranded ends can then 'snap back', making new base pairs so as to reform the hairpins (e). DNA ligase can then covalently rejoin the cut ends (f) to form two intact ends (g). My original model differs from Bateman's in supposing that at stage d extra DNA is synthesized by adding nucleotides to the two shorter chains until they are as long as the longer ones: on that model hairpins would be present only during DNA synthesis, and their replication would be semi-conservative rather than conservative. A newer version postulates that both the cutting and the rejoining are done by a DNA topoisomerase⁹.

sized pieces are not produced simply by cutting up the macronuclear DNA, as I suggested¹, but that new terminal sequences are added, either by DNA splicing using banks of repeated sequences from elsewhere in the genome or by some kind of terminal transferase. The methods used were insufficiently sensitive to show that absolutely no C₄A₄ repeats are present in the cloned micronuclear sequence.

In *Tetrahymena*, the micronuclear sequence from which the rDNA plasmid is derived by gene amplification has now been shown to contain a single C₄A₂ sequence⁵; but it is not yet known whether the tandem C₄A₂ repeats at the ends of the plasmid originate by the repeated duplication of this sequence or by transposition from the tandem C₄A₂ repeats known to exist elsewhere in the genome.

If terminal repeats in *Oxytricha* and *Tetrahymena* macronuclei can be added or lengthened, this might also occur in a similar way in every eukaryote during DNA replication as a means of lengthening the short 5' ends left by the excision of RNA primers (see Fig. 1). Whether this is so or not, it seems likely that elucidating the mechanism of addition of the repeated

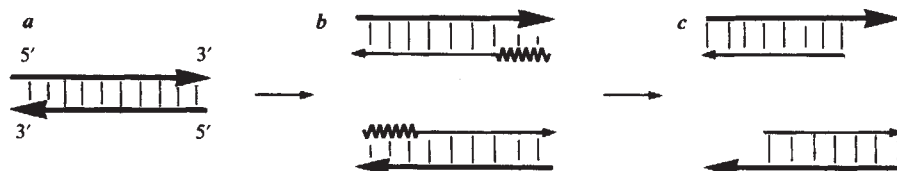


Fig. 1 Replication of a linear DNA molecule lacking special terminal sequences (a) by the standard mechanism involving 5'-terminal RNA primers (b, zigzag lines) would leave unfillable gaps at the 5' ends of the newly made DNA strands when these primers are excised by specific exonucleases (c). If the 5' ends consisted of tandemly repeated sequences, such as C₄A₂ or C₄A₄ found in ciliate macronuclei, then excision of the RNA primers would merely reduce the number of repeats if the primer were equal in length to an integral number of repeats. If the cell has a mechanism for increasing the number of repeats, as King and Yao's results imply for *Tetrahymena*, this could be used to fill in the gap. The mechanism, though unknown, must be distinct from the classical hairpin priming model (Fig. 2) since a sequence containing only Cs and As is inherently unable to fold back on itself to form a self-complementary hairpin, as neither G + C nor A + T base pairs are possible. It even seems possible that the C_mA_n repeats were selected because of their inability to form hairpins and cause potentially harmful cross-linking of the two strands in the manner shown in Fig. 2.

ends to ciliate macronuclear DNA and their role in replication will help clarify telomere structure and function in general.

Earlier proposals for telomere structure mainly stemmed from the hairpin priming^{1,5} model for the replication of chromosome ends (see Fig.2). Although there is evidence for terminal hairpins in several viruses and some evidence also in yeast chromosomes⁶, and in the *Tetrahymena* plasmid (and also at one end of *Paramecium* mitochondrial DNA), the structure of the *Oxytricha* fragments is such that they could not fold back to form hairpins. Telomeres lacking hairpins or inverted repeats might use a nucleotide covalently attached to a DNA-binding protein, as observed in adenovirus⁷ and phage ϕ 29 (ref. 8), as a primer; the covalent attachment of a protein, by preventing the ligation of telomeres, would also explain their stability compared with broken ends just as well as do the hairpin models.

Szostak and Blackburn ingeniously tested the possibility that a simple covalently bonded terminal hairpin could serve as a telomere by constructing an artificial hairpin from part of *Escherichia coli*'s β -galactosidase gene and attaching it to one end of their linear plasmid. This could not be stably replicated in yeast, showing that a terminal hairpin alone is insufficient for replication, as is expected on my original model¹ which also requires specific recognition sequences for an endonuclease (or topoisomerase⁹).

Detailed study of viruses has greatly clarified the basic mechanisms of initiation and chain elongation during cellular DNA replication, because viruses often use different parts of the host cell's complex replication machinery. This may also be true for the end replication of viruses such as parvovirus and vaccinia virus that have terminal hairpins. But since other linear viruses lack hairpins and perhaps use primer proteins, there seem to be several possible mechanisms for the replication of chromosome ends, so there is no substitute for the direct study of eukaryote telomeres that Szostak and Blackburn's new cloning vector now makes possible. It will be particularly important to establish that their yeast 'telomeres', isolated by a purely functional test, really are located at chromosome ends, because unlike the 'ends' studied by Forte and Fangman⁶, they do not 'snap back' rapidly after denaturation.

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Ovarian hormones

New functions for oxytocin?

from R.B. Heap

OXYTOCIN is a hormone that has until recently been associated only with milk ejection and uterine contractions. A series of papers published last year now suggests that oxytocin also plays a part in the control of the oestrous cycle. This discovery may have important implications for contraception research.

The possibility of a wider role for oxytocin first arose in 1959 when it was shown that oxytocin hastens the onset of oestrus in cattle by shortening the life of the corpus luteum¹. Similar effects have since been described in sheep and goats^{2,3}. The hypothesis that oxytocin is involved in the regulation of luteal function is supported by both the increased release of PGF_{2 α} (which is luteolytic) from the uterus in response to oxytocin⁴ and the increase in the number of uterine oxytocin receptors that occurs towards the time of luteal regression⁵. More recently, Tony Flint at Babraham, Cambridge, has shown that the life of the corpus luteum can be prolonged in sheep by active immunization against oxytocin⁶. Measurements of the hormone in peripheral blood further indicate that levels increase with formation of the corpus luteum, and decrease at luteal regression⁷⁻⁹.

Very recently, it has been shown that the corpus luteum itself contains high concentrations of oxytocin^{10,11} and secretes it in response to PGF_{2 α} ¹². In Bristol, Claire Wathes and Ray Swann tested whether the corpus luteum of sheep contains relaxin, like that of the pigs by using a rat uterine strip in an organ bath to monitor activity in luteal extracts. However, they found that the extracts, rather than decreasing the rate of uterine contractions, which would be expected if relaxin was present, increased the contraction rate. Among possible stimulatory substances was oxytocin, and Wathes and Swann went on to show by radioimmunoassay, HPLC and bioassay that the extracts did indeed contain this hormone¹⁰. Wathes and Swann have also shown oxytocin to be present in human¹³ and bovine corpora lutea.

In experiments carried out concurrently with those at Bristol, Flint and Sheldrick found that when sheep were treated with Estrumate (Cloprostenol, ICI 80996, a potent luteolytic analogue of PGF_{2 α}) to induce luteal regression, oxytocin was released into the circulation^{12,14}. This effect could be eliminated if the sheep were ovariectomized or hysterectomized, suggesting that oxytocin is secreted by either the ovary or the uterus. These authors therefore cannulated the ovarian and utero-ovarian veins in anaesthetized sheep, and showed that the ovary can be stimulated by PGF_{2 α} (ref. 12). They have subsequently found that luteal synthesis of

oxytocin can be blocked by hysterectomy and by pregnancy, suggesting that a product of the uterus regulates oxytocin production in the corpus luteum.

Although neither the Bristol nor the Babraham group has yet isolated and sequenced luteal oxytocin, on the basis of cross-reaction with several different oxytocin antisera and evidence from physical and bioassay methods, it seems most likely that the compound is oxytocin or a very closely related peptide. These interesting observations on an ectopic site of oxytocin synthesis in a normal animal open a new era in the study of so-called neurohypophysial hormones. The only other ectopic site of synthesis of neurohypophysial hormones is the Oat cell bronchial carcinoma, which secretes arginine-vasopressin, oxytocin and their respective neurophysins¹⁵. It is interesting that other hypothalamic peptide hormones may be present in gonadal tissues — a peptide resembling luteinizing hormone-releasing hormone has recently been demonstrated in the testis¹⁶.

These findings not only question our ideas about the sites of synthesis of 'brain' hormones, but they may also be relevant to the control of luteal function in the ovary. Research groups in California¹⁷ and Brisbane¹⁸ have recently described inhibitory effects of neurohypophysial hormones, including oxytocin, on steroid synthesis in the testis and corpus luteum. The possibility arises therefore that oxytocin may be the luteolytic substance so long sought in vain in the human corpus luteum. If this is the case, its discovery in the ovary may have important consequences in contraception research. Could it be that oxytocin analogues rather than steroid derivatives offer a new form of contraception for the future? □

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Lymphocyte differentiation

Control of antibody production

from Miranda Robertson

THE differentiation of B lymphocytes is of particular interest from two points of view. To the molecular biologist the singular behaviour of the immunoglobulin genes, which undergo rearrangements that profoundly affect their expression, offers an opportunity to study the basis of eukaryotic gene regulation. From a more practical point of view, the cellular interactions that later control the proliferation and maturation of immunoglobulin-bearing B lymphocytes are crucial to the regulation of immune responses. The problem of gene expression has been approached with cloned DNA; that of immune regulation with cloned cell lines. Some progress on both fronts was reported at a recent meeting in Basel^{*}; and in the latest issue of the *Proceedings of the National Academy of Sciences U.S.A.*¹, Rice and Baltimore report the forging of the first link between cellular and molecular regulation of antibody production.

The gene rearrangements that take place in lymphocytes of the B lineage bring the DNA encoding the variable (V) region of the immunoglobulin molecule, which is transcriptionally silent, into proximity with the DNA encoding the constant (C) region, which is constitutively transcribed^{2,3}. After rearrangement, the complete gene, comprising both V and C segments, is transcribed at a significantly higher rate. It has been suggested² that this amplification may be the combined effect of the upstream promoter of the V segment and the structure of the chromatin in the region of the C segment. Further amplification of the rate of transcription can then be induced by external signals.

In an attempt to elucidate the mechanisms underlying these amplifications, people in several laboratories have been transfecting cells of both lymphoid and non-lymphoid lineages with cloned immunoglobulin genes. Earlier this year, Falkner and Zachau⁴ reported that cloned κ light-chain genes in non-lymphoid cells were not transcribed under the influence of their own promoters, although transcription could be demonstrated if they were placed under the control of a viral promoter. They suggested two possible explanations for this failure — either immunoglobulin gene expression may depend on tissue-specific factors not synthesized in non-lymphoid cells; or alternatively, transcription may depend on chromatin structure in ways that could be influenced by sequences upstream or downstream of the cloned DNA.

In the light of results reported by Richard Maki (Massachusetts Institute of

Technology), the former possibility can be ruled out, at least for heavy-chain expression. Steven Gillies, working in the laboratory of Susumu Tonegawa (Massachusetts Institute of Technology), has used both λ and κ light-chain clones and a $\gamma 2b$ heavy-chain clone to transfect a mouse fibroblast line and finds that the heavy chain is transcribed under the control of its own promoter. The λ and κ transcripts, however, appear for unknown reasons to be longer than those of the myeloma cells in which the genes originated.

Meanwhile, evidence of a different kind increasingly implicates chromatin structure in the regulation of immunoglobulin gene transcription. For example, Robert Perry and his colleague Elizabeth Mather (Institute for Cancer Research, Philadelphia) have shown that the state of methylation of immunoglobulin genes is determined by their chromosomal location. It is a general (though not invariable) rule that DNA sequences are undermethylated in those cells in which they are transcribed. Perry and Mather find, in conformity to this general rule, that the rearranged V-gene segments in plasmacytomas are undermethylated. But unrearranged V genes in the same nucleus, and with virtually identical sequences, are methylated. They accordingly suggest that the formation of a transcriptionally active chromosome domain may depend on the overall DNA context of the region, the lack of methylation serving to stabilize rather than to establish the necessary structure.

The end result of successful rearrangement of the immunoglobulin genes is the expression of an immunoglobulin M molecule on the surface of the B cell; and here begin the problems of cellular immunologists seeking to discover how the humoral immune response is controlled. The nature of the early signals that drive the small resting B cell to the blast stage, at which it becomes susceptible to the signals inducing proliferation and immunoglobulin secretion, is controversial; but the early stimuli can be mimicked *in vitro* by bacterial lipopoly-saccharide (LPS), one of whose effects is to increase still further the rate of transcription of surface immunoglobulin.

Rice and Baltimore¹ have now succeeded in demonstrating LPS inducibility in a cloned light-chain gene introduced into cells of a lymphoid line, and have thus opened an approach to the question of how such signals influence gene expression. At this stage, they are able to conclude only that the sequences necessary for LPS inducibility must lie within 1.5 kb on either side of the coding sequence, and they do not know whether induction is direct or

whether the direct effect is on heavy-chain transcription which in turn regulates light-chain transcription.

In the exploration of the later signals that induce the final maturation of B lymphocytes, attention has focussed increasingly on those B-cell responses that depend on T-cell help, because the development of cloned T-cell lines promises to clarify at last the cellular interactions underlying them^{5,6}.

The isolation of later-acting factors produced by T-cells is now well under way; but if this is making T-B cell interactions clearer, it is certainly not making them simpler. It is now clear, for example, that distinct factors are necessary to induce proliferation of B-cell blasts and to stimulate their maturation into antibody-secreting plasma cells. Factors stimulating proliferation have been isolated from T-cell lines or T hybridomas by Howard *et al.*⁷, Lernhardt *et al.*⁸ and P. Pettersson (Sweden). The factor isolated by Lernhardt *et al.* is known as BRF and is assumed to represent the replication-inducing activity in B-cell replication and maturation factor (BRMF), which was originally extracted from uncloned T cells and induces both replication and antibody secretion⁹.

The attempt by Maureen Howard and her colleagues (NIH) to use a similar (identical?) factor, B-cell growth factor (BCGF), to maintain long-term clones of immature B cells has revealed further unsuspected complexities in B-cell differentiation. She can induce proliferation in B-cell blasts with a combination of BCGF and interleukin-1, which is produced by macrophages; but not with either alone. BCGF is apparently necessary to drive the cell through the early phases of the mitotic cycle, and interleukin-1 for later phases. Even with both these factors, however, the growth of B cells is sluggish and the expectation that they could be used to maintain long-term clones¹⁰ has been disappointed. Hopes are now pinned on two more recently isolated factors: B15-TRF, which may promote later rounds of cell division; and EL-TRF, which resembles biochemically the T-cell growth factor interleukin-2 but acts on B cells rather than T cells. □

Miranda Robertson is an associate editor of Nature.

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Bimodal grain size distribution and secondary thickening in air-fall ash layers

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Investigation of the ashfall deposit from the 1980 Mount St Helens eruption reveals that the deposit is bimodal, the finer population maintaining a uniform grain size distribution away from source, and that the deposit exhibits secondary thickening beyond the 1-cm isopach. Grain size distribution shows that at least 64% of the deposit is finer than 63 μm , far more than is generally supposed for plinian eruptions. An investigation of other ashfall deposits reveals similar grain size bimodality features, which are interpreted as evidence of aggregation of fine particles in the eruption cloud. Our data suggest that aggregation may be a common process during ash fallout from explosive eruptions and that secondary thickening may often occur, providing that other conditions are met.

GRAIN size and thickness variations in pyroclastic fall deposits provide valuable information on the volumes of magma erupted and insights into the mechanisms of explosive volcanism. A major problem in their study is the lack of data on the fine-grained distal parts of pyroclastic fall deposits, which are generally eroded away except in large magnitude eruptions. The complexity of large magnitude events can make interpretation of their distal ashfall deposits problematical. They have been variously interpreted as pre-ignimbrite plinian air-fall deposits¹, co-ignimbrite ashfall deposits^{2,3}, phreatoplinian⁴ and deposits relating to intense littoral explosions generated where hot pyroclastic flows enter water⁵. Criteria for distinguishing these various origins unambiguously have not yet been fully developed.

Most studies of pyroclastic fall deposits only provide data on thickness and grain size characteristics to distances <150 km. Those pre-historic ash layers which have been studied in detail at great distances (>150 km), have always been associated with complex caldera-forming ignimbrite eruptions where interpretation is still controversial¹⁻⁵. Until the 1980 eruption of Mount St Helens the data on distal plinian deposits had been scanty and generally of poor quality. The 18 May activity provided the first detailed documentation of the distal parts of a deposit formed from a moderate-sized plinian eruption⁶⁻⁸. Two major surprises emerge from these studies. First, the air-fall deposit thinned exponentially to 1.0 cm thickness at 180 km, but then thickened to 4.0 cm thickness at 300 km before thinning once more at greater distances⁷. The occurrence of this secondary thickening had been described only once before, from the 1932 eruption of Quizapu volcano⁹. Second, the volume of fine-grained ash produced during the Mount St Helens eruption was much greater than had been generally supposed for plinian eruptions, with 64% of the ejecta deposited within 500 km from source being finer grained than 63 μm .

The St Helens data raise several questions fundamental to the interpretation of distal pyroclastic fall deposits. Is the secondary thickening characteristic of plinian deposits or is it a special and unusual feature of the St Helens eruption? If secondary thickening is common it implies that the conventional tech-

niques of estimating volume of air-fall deposits by extrapolating thickness versus distance plots and assuming an exponential power law decrease in thickness can be in substantial error. In addition, estimates of total grain size distribution in plinian deposits will require revision. Carey and Sigurdsson⁸ attributed the secondary thickening to premature fallout of fine ash due to particle aggregation in the eruption column. Clearly it is of considerable interest to understand the mechanism of particle aggregation and look for evidence which indicates the frequency of occurrence of secondary thickening.

We present here grain size data on several distal air-fall ash layers, including the 18 May 1980 deposit of St Helens. All these deposits show a pronounced bimodal grain size distribution in some localities. In their model for secondary thickening and bimodal grain size distribution, Carey and Sigurdsson⁸ proposed that these were both the consequence of the same fallout mechanism, due to particle aggregation. We will present a case that the bimodality observed in all distal ash layers studied by us is a consequence of this mechanism. The occurrence of bimodality in many ash layers implies that there may have been secondary thickening. We note, however, that other factors, such as the total grain size distribution of the eruption plume, the proportions of crystals, lithics, glass shards and pumice, the prevailing atmospheric conditions and the vapour content of the eruption plume may all be important in producing particle aggregation and secondary thickening. However, the particle aggregation and possibly the secondary thickening seen in the 1980 Mount St Helens activity should not be regarded as exceptional. We also discuss the implications of this interpretation for volume estimates, magma fragmentation processes and the origin of fine distal ash layers.

Methods

All samples were sieved at 0.5 ϕ intervals for coarse ash and then the fine ash was analysed by the electrosensing zone method, using the Elzone Celloscope as described by Muerdter *et al.*¹⁰. The exact techniques varied in detail in the two

laboratories. At Cambridge mechanical sieving was completed down to 90 μm diameter and then the fine fractions were analysed using two different orifice diameters to cover grain diameters down to 4 μm . At Rhode Island samples were mechanically sieved down to 20 μm and then analysed by the same electrosensing zone method for finer grain sizes. Samples from the 1979 Soufriere ash were analysed and showed complementary trends in both laboratories. The close agreement between analyses of standard materials using the settling tube and the electrosensing methods allows us to be confident that the strong bimodality observed in many of the samples studied is not an artefact of our methods.

The 18 May 1980 Mount St Helens deposit

The 18 May activity of Mount St Helens began with a complex series of vertically and laterally directed blasts as the northern side of the volcano failed, generating an enormous landslide¹¹. During the first half-hour the eruption developed into plinian activity with the formation of an eruption column >20 km high which continuously discharged ejecta for 8 h. Pyroclastic flow deposits were generated during the afternoon in the later part of this moderate-sized plinian eruption. The widespread deposits consist of two reversely graded units which in proximal localities are lithic rich at the base, pumice rich at the top and moderately sorted throughout.

On the isopach map of the 18 May plinian deposits drawn by Sarna-Wojcicki *et al.*⁷ and Carey and Sigurdsson⁸, a secondary thickness maximum occurs at 300 km distance. Figure 1a shows three representative grain size distributions (where $\phi = -\log_2 r$ where r is the grain diameter in mm) of samples collected down the dispersal axis. The two populations have been separated visually and the parameters $Md\phi(\phi_{50})$ and $\sigma\phi[(\phi_{84} - \phi_{16})/2]$ determined from cumulative frequency curves. The coarse population shows a systematic decrease of $Md\phi$ with distance from source (Fig. 2a) whereas the fine population shows no regular variation, maintaining relatively uniform values of $Md\phi$ irrespective of sample location.

Carey and Sigurdsson⁸ have developed a quantitative model for the fallout of the Mount St Helens ash deposit predicting thickness and grain size variations. The model uses the measured wind velocity profile in the atmosphere on 18 May and considers the fallout of ash particles with an initially random distribution through the volcanic plume. Their initial model, assuming all particles fall freely without any interaction, reproduces the proximal (<180 km) decrease in ash thickness satisfactorily, but fails to explain the secondary thickening, fine grain size and component proportions of the distal deposit (Fig. 3). The problem can be illustrated simply by reference to the fine grain size population. If these particles fell freely in the observed atmospheric wind profile from the observed height of the plume they would be blown to distances of thousands of kilometres before being deposited. Carey and Sigurdsson⁸ proposed that the fine ash fell prematurely in particle aggregates or clumps. By modelling the fallout of particles finer than 63 μm as clumps with a settling velocity of 0.35 m s^{-1} , Carey and Sigurdsson reproduced the secondary thickening (Fig. 3).

Aggregation of fine ash particles during the Mount St Helens eruption has been demonstrated convincingly by Sorem¹² who observed that the ash fell like snow, landing as clumps of typical diameter 250–500 μm . We propose that the fine grain size population in the Mount St Helens ash is due to fallout of these particle aggregates and that the coarse population is due to fallout of individual freely falling particles.

The 1979 Soufriere ash, St Vincent

The 1979 explosive eruption of the Soufriere volcano, St Vincent, produced a series of fine grained and poorly sorted ashfall deposits. Eleven episodes of explosive activity occurred in short intense periods lasting 5–22 min between 13 and 26 April 1979^{13,14}. The activity is interpreted as phreatomagmatic with

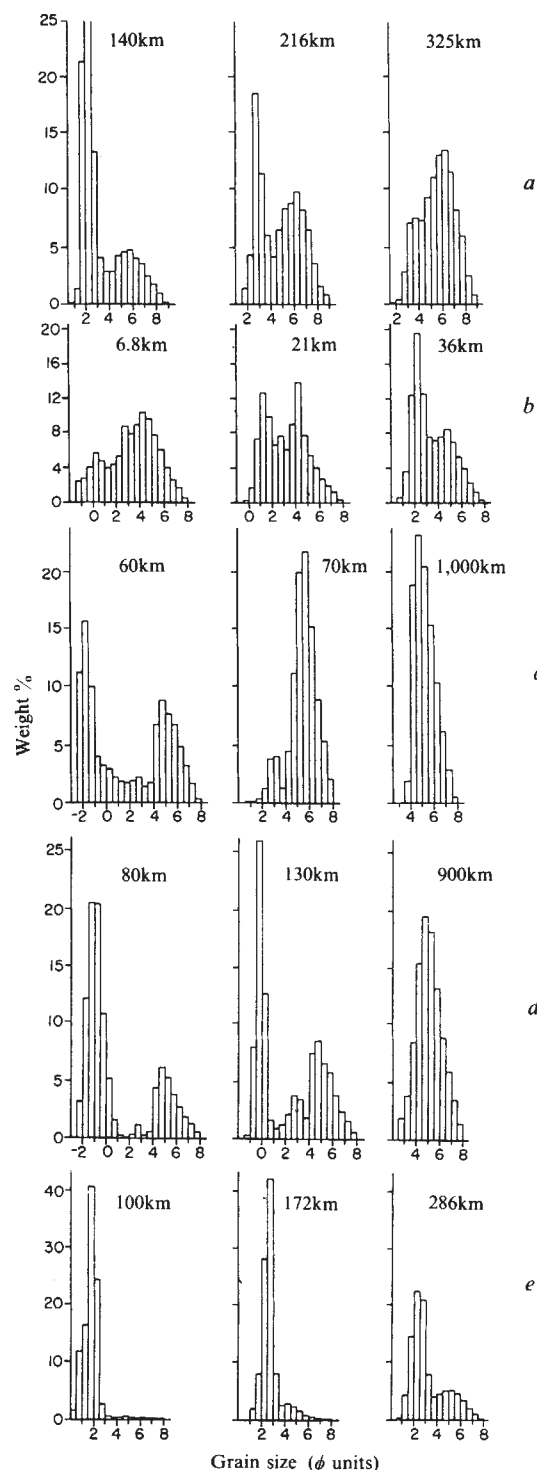


Fig. 1 Grain size distributions at various distances downwind of *a*, 18 May 1980 Mount St Helens deposit; *b*, 26 April 1979 Soufriere, St Vincent deposit; *c*, 7,000 yr BP ash of Mount Mazama; *d*, 12,000 yr BP ash of Glacier Peak; and *e*, 3,500 yr BP ash of Mount St Helens.

rising basaltic andesite magma interacting with water in the crater lake of the Soufriere¹⁴. Several of the ash layers show pronounced bimodality. The most completely documented ash layer formed on 26 April¹⁵, is bimodal (Fig. 1b) and shows a systematic decrease of $Md\phi$ with distance from source (Fig. 2b) in the coarse population, but no systematic variation in the fine population. A substantial proportion of the ash fell as 1–7 mm diameter accretionary lapilli. Some of the accretionary lapilli are themselves bimodal. We interpret the bimodality of

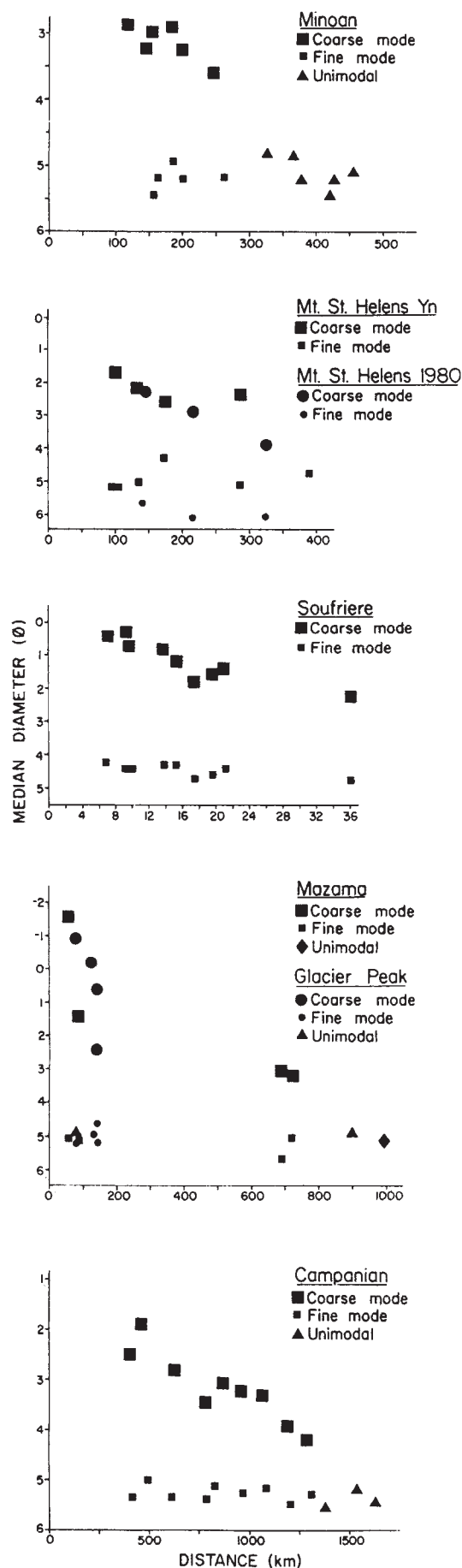


Fig. 2 Median diameter against distance plots for the ash layers analysed.

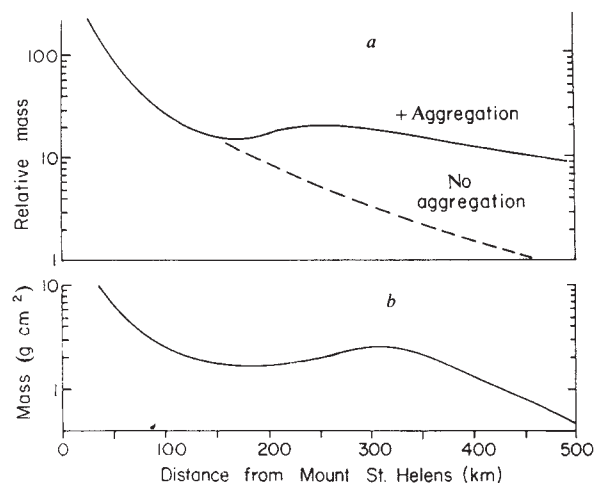


Fig. 3 Mass against distance relationship for the computer-predicted deposit (a) and the observed 18 May 1980 deposit (b). Data for the observed deposit are from WSDNR Open File Report 80-12.

the lapilli as a consequence of aggregation of fine ash before their formation. In a plot of $Md\phi$ versus $\sigma\phi$ (Fig. 4) the close similarity between the fine size populations in the St Helens 1980 and Soufriere deposits suggests that they have the same origin.

North American ash layers

During a study of widespread distal ash layers found in the north-west USA and Western Canada, the following deposits were investigated:

- (1) The 1,200 yr BP Chiwawa ash of Glacier Peak, Westgate¹⁶, identified as layer B by Porter¹⁷.
- (2) The 7,000 yr BP ash of Mount Mazama; Powers and Wilcox¹⁸.
- (3) The 1,890 yr BP ash of White River (northern lobe), Lerbekmo *et al.*¹⁹.
- (4) The 2,500 yr BP ash of Bridge River, Nasmith *et al.*²⁰.
- (5) The 3,500 yr BP Mount St Helens Yn ash, Mullineaux *et al.*²¹.

Samples at various distances from source were analysed for their grain size distribution (Fig. 1c, d, e). The Bridge River and Mount Mazama ashes are bimodal in proximal localities. At increasing distances from source the $Md\phi$ values of the fine population remain uniform, while those of the coarse population become finer, the two populations eventually merging to form a unimodal deposit. This feature is illustrated by Mount Mazama data in Figs 1 and 2.

The more closely spaced sampling of the Mount St Helens Yn ash reveals some interesting features of the deposit. From 100 to 172 km from source, the $Md\phi$ of the coarse population fines systematically from 1.73 ϕ to 2.6 ϕ , while that of the fine population varies randomly between 5.2 ϕ and 4.35 ϕ (Fig. 2a). Between 172 and 388 km the overall grain size remains relatively uniform (Figs 1e and 2a) and the percentage of the sample contained within the fine population increases from 13.5 to 48.5%. The May 1980 Mount St Helens deposit shows a marked increase in fines and only gradual changes in grain size within the area of secondary thickening, that is, 200–400 km from source (Fig. 1a and ref. 8).

The proximal Glacier Peak samples are trimodal, comprising a coarse population which, as in the other ashes, becomes finer away from source, and a stationary fine population. The third, intermediate population is formed by a maximum of crystals occurring within this size range. The coarse and fine populations are shown on Fig. 2d. The White River samples are all bi- or trimodal, the intermediate population again being formed by a crystal maximum. Figure 4 illustrates the close similarity in $Md\phi$ and $\sigma\phi$ values shown by the fine population of all the

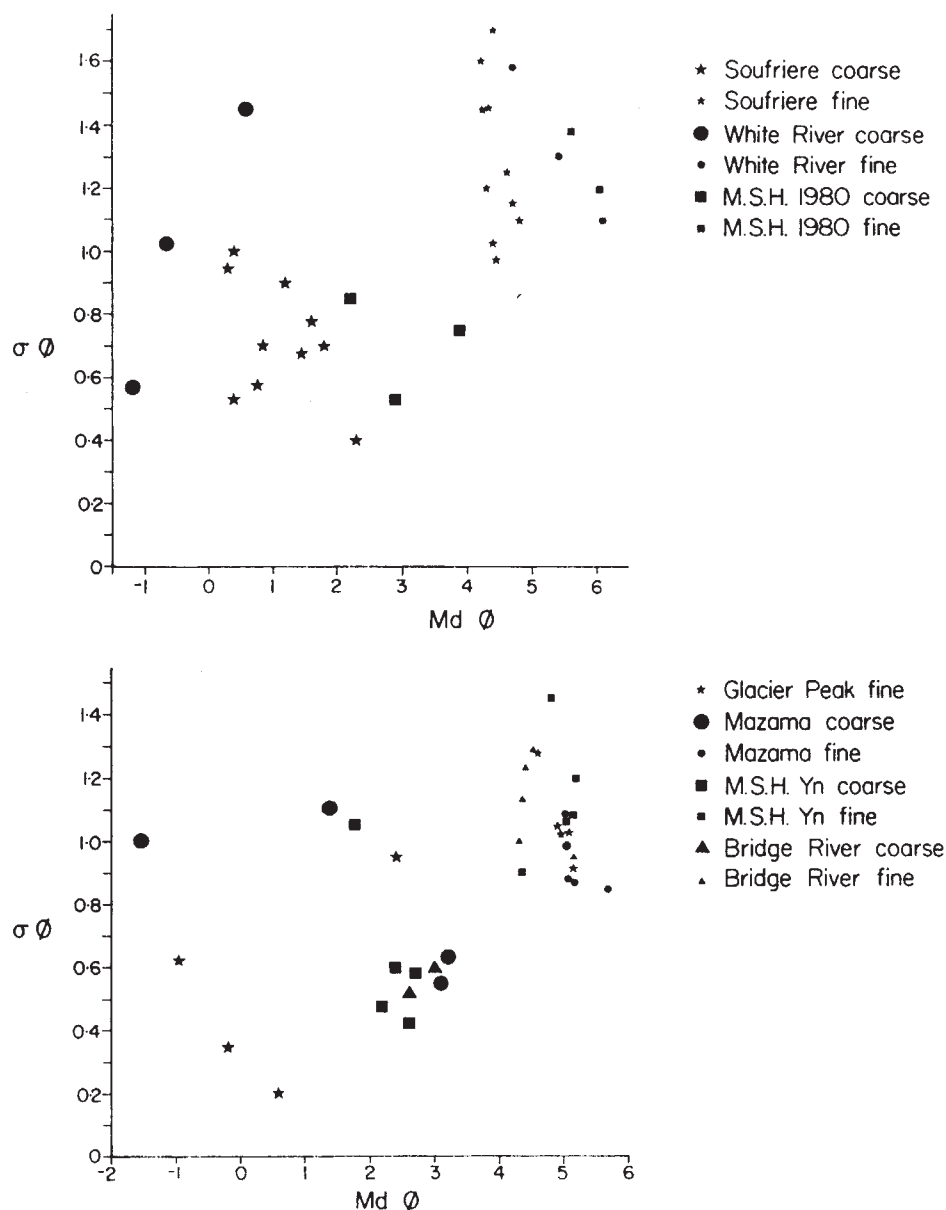


Fig. 4 $Md\phi$ versus $\sigma\phi$ plots for the ash layers analysed.

ash layers in this study, including the White River ash. All these ash layers exhibit bimodality, with a fine population whose grain size distribution is not affected by distance from source. We interpret the fine population as a consequence of aggregation of fines, causing premature fallout. The presence of bimodality in ashes produced by eruptions of different styles and magnitudes suggests some uniform process of fines aggregation.

Deep sea ash layers

Sparks and Huang³ presented a detailed study of two deep-sea ash layers, the Campanian Y5 ash layer and the Minoan ash layer. These deposits were found to be bimodal, displaying a coarse population which became systematically finer away from source and a fine size population which remained uniform, irrespective of distance from source (Fig. 2d, e). Cornell *et al.*²² have presented new data on the Campanian Y5 and re-interpreted the results. Using a computer program to model ash fallout from a variety of possible eruption column heights and wind profiles they assess which conditions would give rise to the observed deposit. They conclude that the coarse population was formed by fallout of individual particles from a high plinian eruption column. Cornell *et al.*²² have been able to constrain the upper level of significant ash transport to between 25 and

35 km. The modelling also demonstrates that if all the fine ash fell as individual particles from such a high column, no significant deposition of ash $<22\ \mu\text{m}$ in diameter would occur within the 1,600 km area studied. Thus the fine population cannot be attributed to the free fallout from a high column. Cornell *et al.*'s modelling constrains the transport of ash in the fine population to below 3 km height if the ash particles are regarded as falling individually. They point out, however, that the great regional variability in low-level winds over the Mediterranean argues against such a low-level transport model for the Campanian ash. The direction of low-level winds over Italy is also inconsistent with the observed dispersal. Thus Cornell *et al.*²² interpret the fine mode as a result of fine ash transported in a high altitude eruption column, resulting in settling of 200 μm aggregates. The eruption column and ash plume were fed by a combination of plinian activity and convective uprise of fine ash elutriated from pyroclastic flows near the vent, rising to great heights.

The uniformity of modal values shown by the fine population irrespective of distance from source (Fig. 2) and the similarity of these values to those shown by the fine populations of the other ash layers studied, leads us to prefer a model of particle aggregation of fines to explain the bimodality in both the Campanian Y5 and the Minoan ash layers.

Conclusions

Bimodal grain size distribution is a common feature in many distal ash layers and in some proximal deposits, such as the Soufriere and White River deposits. The grain size distribution of the fine population is remarkably similar in the examples we have studied (Fig. 4) although both the magnitude and style of the eruptions and environments of deposition differ substantially from case to case.

We propose that the bimodality can be attributed to premature fallout of fine ash aggregated in the eruption column. The uniformity of size characteristics of the fine population provides evidence for a single process operating in all these eruption columns. This process must be independent of the style and magnitude of the eruption, occurring in a small phreatomagmatic eruption of the Soufriere and in huge ignimbrite eruptions such as the 7,000 yr BP Mazama eruption. We suggest that all that is required is a substantial quantity of fine ash. It is not appropriate here to speculate in detail on the aggregation mechanism although we suspect that electrostatic charging and vapour condensation in the eruption column (L. Wilson, personal communication) may provide a plausible solution.

Sparks and Huang³ attributed the bimodality of the deep-sea ash layers to the occurrence of two phases in these eruptions: a plinian phase producing coarse particles falling out from a high eruption column and an ignimbrite phase producing a fine size population from a low eruption column. We are, however, impressed by the similarity between these deep-sea ash layers and the data from ash layers on land. This, together with the constraints placed on the method of deposition of the fine population by the modelling of Cornell *et al.*, leads us to interpret the fine population as fallout of aggregated fines from high eruption columns.

Our interpretation of the Mount St Helens data shows a direct link between secondary thickening and bimodality, both being formed by the same process: premature fallout. If this is accepted then the occurrence of bimodality in the North American ash layers implies that secondary thickening may also be a common process in ashfall deposits, provided that certain other necessary conditions are met. Three of the deposits (White River, Mount St Helens Y and Bridge River) are of plinian type. Secondary thickening and bimodality may both be typical characteristics of plinian deposits.

There are some fundamental implications for volcanology if these ideas are correct. First, secondary thickening can occur in plinian and other types of air-fall deposits and is not a peculiarity of the Mount St Helens eruption. The phenomenon has only rarely been recognized before, because the fine distal deposits are easily removed. Thus most studies of plinian deposits at distances typically <150 km will not detect such a feature. Clearly the conventional way of estimating volumes of deposit by extrapolating the near source exponential thinning of deposits to large distances must be called into question.

A second implication is concerned with the degree of magma fragmentation during plinian eruptions. Most estimates of total grain size distribution involve integrating and averaging the

grain size distributions over the entire area of the proximal 'exponential' part of the deposits. In the case of St Helens Yn ash layer, the Bridge River, Mazama and White River deposits this procedure would neglect the significant volume of distal fine ash. The data presented here suggest that the volume of fine ash produced in plinian eruptions is much larger than previously supposed. Walker's study²³ of the crystal content of the Taupo plinian deposit in New Zealand led him to conclude that 80% of the ejecta was deposited at distances >220 km, implying an enormous volume of fine ash. Walker's volume estimate of the Taupo deposit was more than double that assuming an exponential thickness decrease extrapolated to large distances. Similarly, Carey and Sigurdsson²⁴ have shown on the basis of crystal-glass fractionation, that almost half of the erupted tephra from the ashfall phase of the Roseau eruption on Dominica was transported as fine ash beyond the area of principal air-fall deposition.

Our data raise more questions than they solve. Much more work is required before a definitive picture can emerge. Meanwhile we comment on some of the implications: (1) What is the mechanism of aggregation and what physical parameters control its occurrence and efficacy? (2) What is the significance of large volumes of distal fine ash in some plinian deposits? Does this signify great violence as suggested by Walker for the Taupo deposit? This hypothesis is complicated by the fact that the Mount St Helens eruption was not a particularly large eruption (20-km high column) yet it also shows a great abundance of fines. Does the fine ash imply a phreatomagmatic element to these eruptions? If this were so we would expect to find examples of plinian deposits which lacked fines and secondary thickening. On the other hand all plinian deposits could show this feature, undetected before for reasons we have already mentioned. (3) How do these data affect the status of co-ignimbrite ashfall deposits? The observation of bimodality can no longer be regarded as good evidence for such deposits. However, there are other lines of independent evidence to support this concept^{2,3,25,26}. Izett¹, in describing North American tephra layers, clearly views all distal ash layers as pre-ignimbrite plinian deposits. Certainly the evidence reported here supports the idea that pre-ignimbrite plinian activity will produce much more fine ash than was generally supposed. However, it is difficult to see, at least on current models of large magnitude eruptions, why there should be a relationship between the volume of plinian deposit and ignimbrite as is indirectly implied by Izett's assertion. If such a relationship were proven then reassessment of eruption models would be necessary. Although volcanological considerations still support the idea that many of these ash layers have a substantial co-ignimbrite component, the plinian component may well be much larger than originally supposed and distinguishing the two origins by grain size characteristics alone may prove impossible.

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Distinct organizations and patterns of expression of early and late histone gene sets in the sea urchin

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*The set of histone genes that are active late in embryogenesis of the sea urchin (*Strongylocentrotus purpuratus*) are present in 5–12 copies per genome and, unlike the clustered, tandemly arrayed early histone genes, are dispersed and irregularly arranged. Late H2B gene expression is activated by events accompanying fertilization and its mRNAs are first detectable by as early as 6 h of development (16 cells) and increase only slightly in amount during the period of rapid cleavage between 6 and 14 h. However, during the short interval between 14 and 16 h, while the amount of early histone mRNA is declining, there is a >15-fold burst in the rate of late H2B mRNA accumulation.*

THE sea urchin synthesizes several sets of histone proteins during the course of its early development. Until the late blastula stage, embryonic chromatin contains almost exclusively early histone proteins encoded by tandemly repetitive early histone genes. In subsequent embryonic development, the synthesis of these early forms is curtailed and replaced by the synthesis of a second set of histone proteins^{1,2} translated on templates differing from the early histone mRNAs in both size and sequence^{3–6}. Such late histone templates are therefore the products of a distinct set of structural genes. By the gastrula stage, late histone mRNAs account for >95% of total histone mRNA synthesis⁶ and embryonic chromatin contains a significant proportion of electrophoretically distinct late H1, H2A and H2B proteins⁷. Synthesis of late histones results in a progressive increase in this proportion, culminating in an almost complete dilution of chromatin-associated early forms in the pluteus larva⁷.

The switch from early to late histone gene expression constitutes a dramatic example of differential gene regulation during embryogenesis. Furthermore, programmes of histone gene expression are nearly identical in widely diverged Echinoids (*Strongylocentrotus purpuratus* and *Lytechinus pictus*)^{3,6,8}, suggesting that the switch has an important role in sea urchin development. We report here the molecular cloning and characterization of late histone genes. We have used a specific late histone H2B DNA sequence as a DNA–RNA hybridization probe to determine the time of onset of late gene expression and to estimate the relative amounts of late histone H2B mRNA at different stages of embryogenesis.

Purification of late histone mRNAs and phage cloning

Late histone genes were selected from a λ phage library by using probes made from late histone mRNAs purified in the following way. RNA was extracted from gastrula-stage embryos, whose histone mRNAs are mostly of the late variety⁶. The RNA was hybridized to cloned early histone DNA covalently coupled to cellulose (described in Fig. 1 legend). Late H2B and H4 histone mRNAs were purified by thermal elution and gel electrophoresis in sufficient yield to be radio-labelled *in vitro* and subsequently used as hybridization probes in the screening of phage libraries.

The thermal stabilities of hybrids formed between late histone mRNAs and early histone genes are substantially lower than those of the homologous early mRNA–early gene hybrids⁶. We therefore predicted that the late mRNA–late gene hybrids would be correspondingly more stable than the late mRNA–early gene combination. Accordingly, we chose hybridization conditions that would select relatively rare late histone sequences among the very abundant early genes. Radiolabelled late H2B and H4 mRNAs were used separately to screen a λ phage recombinant library of *S. purpuratus* DNA (see Fig. 1 legend). From this screening we obtained four putative late histone clones, designated λ SpL1 to λ SpL4.

Identification of clones containing late histone sequences

All four clones formed very stable hybrids with late histone mRNAs (Fig. 1). The T_m values of these are $\geq 75^\circ\text{C}$ when measured in $0.1\times\text{SSC}$. This melting profile is consistent with perfect or near-perfect base pairing of hybrid structures if we assume a length (500 nucleotides) and a base composition (56% G+C) similar to those of early histone genes^{9,10}. Clone L1 hybridizes with H4 and H2B mRNAs, clone L2 with H4 mRNA and clone L4 with H2B mRNA. Clone L3 hybridizes with H2B mRNA and additional RNA bands which cannot be positively identified solely on the basis of these data. In each case, the hybrids formed between L1, L2, L3 and L4 and late histone mRNAs are more stable than those they form with early mRNAs. In a reciprocal manner, the early histone gene control DNA forms more stable hybrids with early RNA than with the corresponding late gene transcripts. We conclude that the cloned DNA segments λ SpL1–4 contain late histone gene sequences.

From Fig. 1 it is also evident that hybrids formed between late histone sequences and late mRNAs show different melting behaviour. For example, two electrophoretically distinct forms of late H2B mRNA hybridize to clone L4. The hybrids they form with L4 DNA show quite distinct thermal stabilities (Fig. 1f). This suggests that classes of histone mRNA (for example, H4) comprise several members differing from each other in both size and nucleotide sequence.

Early histone mRNAs, identified by their electrophoretic mobility, can also be seen in Fig. 1. The hybrids they form with the cloned late DNA sequences show considerably reduced T_m s ($10\text{--}25^\circ\text{C}$ below those of the most stable hybrids). The presence of such early histone gene transcripts in gastrula RNA is to be expected because early histone mRNAs continue to be synthe-

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sized at a low rate as late as prism and pluteus stages of embryonic development⁶.

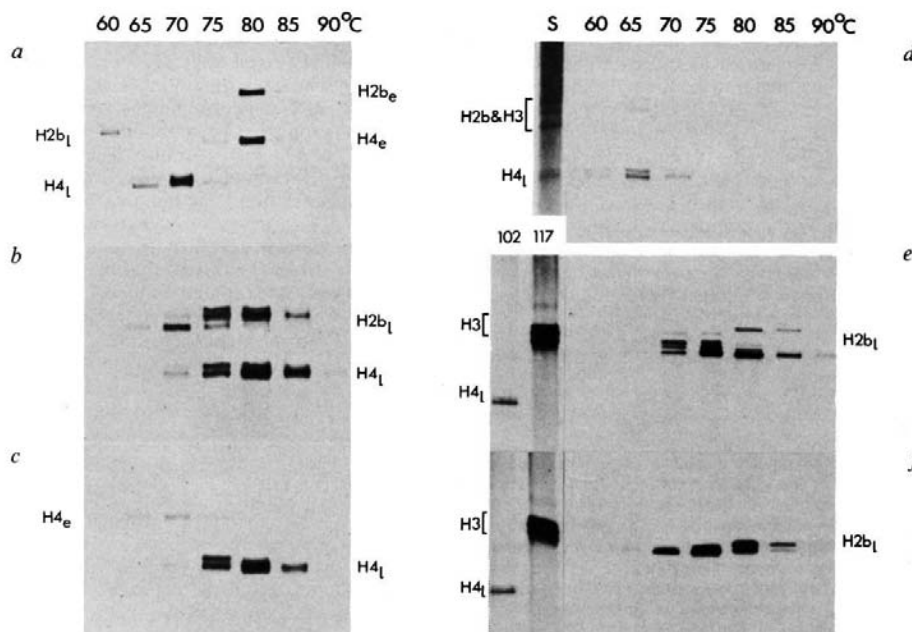
Organization of histone sequences on cloned segments of DNA

We determined the locations of late histone coding regions on the cloned DNA with successively refined restriction enzyme mapping techniques. The resultant maps are shown in Fig. 2. Attempts to determine the order of the *Eco*RI fragments in clone L4 were hindered by the unusual length (~25 kilobases, kb) of the cloned segment and the resultant complexity in the array of partial digestion products. DNA sequencing of small,

hybridizing restriction fragments established the precise borders of the coding regions, the polarities of transcription and unequivocally identified individual histone coding regions (Fig. 2).

Two major results are evident from the mapping studies. First, in marked contrast to the organization of early histone genes, the cloned members of the late histone gene set are neither tightly clustered nor regularly arranged. Second, no common fragments could be discerned in the restriction maps of any of the four clones, indicating that the DNA segments do not overlap. Therefore, the late histone genes described here are organized as isolated single genes or as closely linked pairs which are themselves isolated on large segments of chromosomal DNA. The limits (maximum linkage distances)

Fig. 1 Measurement of the thermal stability of hybrids formed between late histone gene clones and late histone mRNAs. Segments of sea urchin genomic DNA containing sequences homologous to purified late histone mRNAs were selected as follows. RNA was extracted from gastrula-stage embryos with guanidinium hydrochloride¹⁹. Histone mRNAs were purified by hybridization to histone DNA immobilized on cellulose^{6,20}. The hybridization reaction was carried out in 0.66 M NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 0.1% SDS and 50% formamide, for 16 h at 37 °C. The DNA-cellulose was then washed repeatedly in 0.1×SSC (15 mM sodium chloride, 1.5 mM sodium citrate), 0.1% SDS at 37 °C to remove the RNA nonspecifically bound to the cellulose. Histone mRNAs were purified further by stepwise thermal elution of hybridized RNAs followed by electrophoresis of RNAs collected at each temperature step on a polyacrylamide gel. Histone DNA-cellulose was suspended in 0.1×SSC, 0.1% SDS and incubated at 45 °C for 5 min. The DNA-cellulose was collected by centrifugation and the RNA in the supernatant was precipitated with ethanol after the addition of 10 µg of carrier tRNA. Similar cycles of thermal elution were repeated at 5 °C intervals up to 90 °C. The thermal elution step eliminated an otherwise substantial contamination by early histone mRNAs resulting from the selective enrichment of low abundance early mRNAs by early histone DNA cellulose (see below and panel a). The RNA samples were then electrophoresed on a 5% polyacrylamide gel containing 7 M urea^{6,21}. RNAs were visualized by staining with ethidium bromide, excised and recovered from gel slices by overnight incubation in 0.6 M LiCl, pH 5.8, 0.2% SDS at room temperature²². The eluate was passed over siliconized glass wool and the RNA precipitated with ethanol. Purified late H4 and late H2B mRNAs were partially cleaved with base (50 mM Tris, pH 9.5, 65 °C for 15 min) and the resultant 5' OH groups labelled with [γ -³²P]ATP using polynucleotide kinase²³. These radiolabelled RNAs were then used to screen 50,000 phage recombinants (the equivalent of a single genome with an 80% probability) from a library of sea urchin genomic DNA fragments cloned in Charon 4 phage^{24,25}. Conditions of hybridization were chosen to favour the formation of the homologous late histone mRNA-late gene hybrids over the more frequent (because of early gene dosage) late mRNA-early gene interactions. The filters were preincubated in hybridization buffer (50% formamide, 4×SSC, 50 mM sodium phosphate pH 7.0, 0.025% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone) for 12 h at 37 °C. The annealing reaction was carried out in hybridization buffer containing ~50,000 d.p.m. per ml of radiolabelled histone mRNA at 57 °C for 18 h. Filters were then washed with 0.1×SSC, 0.1% SDS at 37 °C for several hours. Separate screenings with H4 and H2B mRNA yielded a total of four positive clones, designated λ SpL1 to λ SpL4. DNA was isolated from the four clones²⁶, immobilized on nitrocellulose filters⁶ and allowed to react with ³H-labelled gastrula-stage RNA. Hybridizing RNAs were recovered by thermal elution cycles as described above. As a control, separate filters containing portions of the early histone gene repeat unit, pSp102 (containing H1, H4 and H2B genes), pSp117 (containing H2A and H3 genes) and pCO2 (containing all five histone coding regions)^{9,27} were treated similarly. mRNAs eluted from such control filters provided electrophoretic markers for early and late mRNAs⁶. RNA fractions were electrophoresed on polyacrylamide gels (see above) which were subsequently fluorographed²⁸ and exposed at -80 °C to preflashed XR-5 film (Kodak) for 3 weeks. Similar regions of the autoradiograms are shown in panels a-f. The numbers above the lanes indicate elution temperatures. The approximate positions of migration of various early and late histone mRNAs are shown on either side of the panels. Unreacted RNA supernatants recovered from hybridization reactions (an example is shown in lane S, panel d) were included in all gels as standards for the relative positions of migration of the various mRNA species. Lanes 102 and 117 (panels e and f) show RNAs eluted from the plasmids pSp102 and pSp117. Note that two different RNA preparations, obtained from approximately the same stage of development but from different sea urchin matings, were used in the experiments. One preparation was used for experiments shown in panels a, b and c, while a second was used for those shown in d, e and f. As is apparent from the autoradiograms, these RNA preparations differ in the amount of residual early histone mRNA sequences they contain. Panels a and d represent control experiments showing RNA species eluted from early histone DNA-containing plasmids pSp102 (a) and pCO2 (d). Panels b, c, e and f are thermal elution profiles of RNAs hybridizing to putative late histone clones pSpL1 to pSpL4, respectively.



of 'loose clustering' are set by the positions of the late histone sequences on the cloned DNA segments. For example, the H4 gene in clone L2 lies near the centre of a large DNA segment. The minimum distance between this gene and its nearest neighbour histone gene is 10 kb on one side and 8 kb on the other. Similarly, at least 1 kb on one side and 15 kb on the other must separate the H4-H2B late gene pair in clone L1 from nearest-neighbour histone sequences.

Not only do the gross genomic organizations of cloned early and late histone genes differ, but so do the genetic fine structures of the early and late clusters. Thus, the finding that the H2B and H2A coding sequences on clone L3 lie adjacent to one another and are transcribed in opposite directions contrasts with the gene order (5'-H1-H4-H2B-H3-H2A-3') and common direction of transcription of the genes in the early repeat unit. No unique late gene order is evident in the late clones. For example, in contrast to L3, L1 shows an early-like gene order and direction of transcription, the H4 gene being adjacent and upstream from the H2B.

Restriction enzyme sites located in the DNA immediately flanking the coding regions are dissimilar in the different clones, even when homologous genes are compared. Therefore, flanking sequences are not tightly conserved among the set of late histone genes. Within comparable coding sequences such as the H2B genes of clones L1, L3 and L4, some common enzyme sites can be found (Fig. 2). Nevertheless, DNA sequencing has revealed extensive differences between all three H2B genes (our unpublished results).

Late histone gene expression

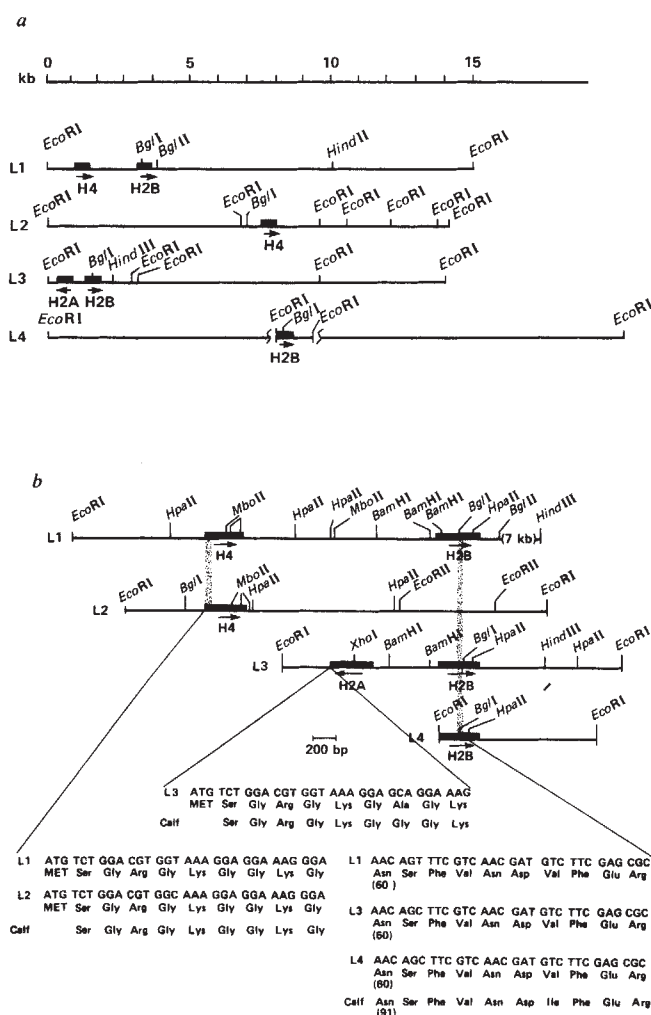
Late H2B mRNA hybridizes with early H2b genes at a non-stringent criterion, but the extent of the reaction is low (our

unpublished observations and see ref. 6). Thus, sequences derived from a late H2B gene should, in appropriately stringent conditions of hybridization, selectively react with other late H2B genes and their transcripts. To test this, we radiolabelled a coding segment of the cloned late H2B histone sequence from clone L1 and allowed it to react with RNA on blots of whole-cell RNA from a series of developmental stages ranging from egg to pluteus. In a parallel experiment, the analogous early gene probe was used in the hybridization. Autoradiograms of the resultant blots (Fig. 3) reveal that the early and late H2B histone gene probes do indeed react specifically with their respective transcripts.

The results of this experiment reveal several important features of early and late histone gene expression. Early H2B mRNA, present in relatively low but easily detectable quantities in the unfertilized egg, increases significantly in amount after the 16-cell stage. By 200 cells (12 h) it reaches a peak and then decreases rapidly, leaving only a small amount of early H2B mRNA in the pluteus larva (72 h). A similar developmental profile has been obtained previously by RNA electrophoresis¹¹ and by DNA titration methods¹².

In contrast, late H2B transcripts are not detected in the unfertilized egg but are observed as early as the 16-cell stage. Thus, this gene is activated by fertilization or events shortly thereafter. Densitometer scans of the gel shown in Fig. 3 (data not shown) reveal that between the 16-cell stage and the 250-cell stage (6 and 14 h post-fertilization, respectively) late H2B mRNA per embryo accumulates steadily to a level about fivefold higher. However, in the subsequent 2 h, the level of late H2B mRNA rises dramatically to about 20-fold over the 16-cell stage level. Thus, in the brief interval between 14 and 16 h of development the rate of accumulation of late H2B

Fig. 2 Gene organization in late histone gene clones. We initially confirmed the coding capacities of the four late histone gene clones by an experiment in which DNA samples from the four late histone clones, prepared as described in ref. 26, were radiolabelled by nick translation²⁹ and hybridized to known fragments of early histone DNA, each containing one of the five histone coding sequences. These fragments were resolved by electrophoresis and transferred to nitrocellulose. The autoradiograph of the blotted gels (not shown) demonstrated that clone L1 contains sequences coding for late H4 and H2B histones, clone L2 codes only for late H4 histone, clone L3 codes for H2A and H2B histones and clone L4 codes for a single H2B histone. The presence of H2A coding sequences on late clone L3 is consistent with its ability to hybridize to several mRNAs other than H2B (Fig. 1e). We mapped the positions of these coding sequences using standard techniques. DNA was digested with various restriction endonucleases alone and in combination; the products were electrophoresed on agarose gels³⁰, transferred to nitrocellulose and hybridized with purified ³²P-labelled histone mRNAs shown previously to be homologous to the cloned DNA segments. *EcoRI* sites were located on the DNA molecules by analysis of the array of sizes of fragments that was obtained on partial digestion of the DNA. Segments of the phage inserts found to hybridize to the late histone mRNAs were subcloned in pBR322 or pBR325³¹ and restriction enzyme cleavage sites of many different enzymes were mapped in these fragments. We show here the long-range maps of the entire λ phage inserts (a) and the more detailed short range maps of the subcloned fragments (b). The broken lines around the H2B gene in the long range map of clone L4 indicate that the exact location in the phage insert of the 1.3-kb *EcoRI* fragment containing the H2B gene has not been determined. Late histone gene coding regions were unequivocally identified and their polarities of transcription determined by DNA sequencing (b). Restriction enzyme fragments known to contain regions homologous to particular late histone mRNAs were end-labelled and sequenced³². In each case a fragment was chosen so that the orientation of the histone gene coding region could be established in relation to the locations of restriction enzyme cleavage sites. This provided the relative polarities of transcription of the various genes. The DNA and derived amino acid sequences of segments (indicated by stippling) of the four cloned late histone genes are shown next to amino acid sequences of calf thymus histone proteins previously determined by direct sequencing³².



mRNA increases by more than 16-fold. In later stages, the amount of late H2B mRNA shows little change.

Number and arrangement of late histone sequences in the genome

We performed a series of experiments to assess the organization and number of late genes in the sea urchin genome. Plasmids containing late H2A or H2B coding regions derived from λ clone L3 were digested with several restriction enzymes, and known amounts of DNA were electrophoresed in parallel with *Bgl*/II-digested genomic DNA. The restriction enzyme *Bgl*/II does not digest the early histone gene repeat unit, with the result that early gene clusters after digestion will remain as high molecular weight fragments at the upper limit of electrophoretic resolution. Sequences outside the early repeat unit, including late histone genes and orphans¹³ (the latter defined as early histone sequences located outside the major repeat unit), can thus be resolved without the interference of a hybridization signal from the early repeat.

To test our ability to distinguish late genes from early gene orphans, strips from a single genomic Southern transfer were hybridized in stringent conditions with early or late H2A probes. The autoradiographs are shown in Fig. 4A. The late gene copy-number standard in lane *b* hybridizes with the late H2A probe whereas the same late gene standard does not hybridize at all with the early H2A probe (lane *d*). An analogous experiment was performed using early and late H2B probes

with the same result (data not shown). Thus, when these late probes are annealed with genomic blots, the hybridizing bands are expected to be late gene fragments. The converse is expected for early probes. Such differences in the specificity of the early and late probes is seen in the case of the H2A probes in lanes *a* and *c*. There is little or no overlap in the sizes of the *Bgl*/II fragments that hybridize with the early as compared with the late H2A probe. There are 10–12 distinct late gene fragments, none of which hybridizes more intensely than the standard which represents two copies per genome.

A more extensive examination of the early and late genomic fragments containing H2B sequences is seen in Fig. 4B. Here we examined the genomic pattern of DNA from five individual sea urchins. In each case there is little or no overlap in the sizes and patterns of hybridization of the early versus late bands. Furthermore, the early bands, which represent orphon gene segments, vary in both number and hybridization intensity from individual to individual, whereas the number of late gene bands (10–12) and their hybridization intensities seem to be relatively constant between individuals. From a hybridization standard reconstruction, we also estimate that most of the late gene bands are single copy (data not shown). The significance of the late gene polymorphism between individuals is unclear because *S. purpuratus* exhibits rather extensive restriction site polymorphism¹³.

A significant hybridization signal is obtained at the upper size limit of the gel blot when either late gene probe is used. This is probably a consequence of the very large number of early genes migrating in that region of the gel. At least 400 early genes in a single band are expected to result in a strong signal, even when the relatively nonhomologous late genes are used as probes. In contrast, the presence of solitary early gene orphans in genomic fragments should not be detected by late gene probes in the hybridization conditions used.

From the data of Fig. 4, we conclude that the genomic fragments rarely contain more than one or two copies of late H2A or H2B sequences. Thus, there are about 10–12 different late H2B or H2A gene segments in the DNA of a single individual. Few fragments hybridize to both late H2A and H2B probes, suggesting that the late H2A and late H2B genes are not frequently located close together. Finally, as the late histone gene family exhibits extensive restriction fragment polymorphism, many bands may represent polymorphic alleles. Thus, there may be as few as five to six copies of each late H2B and H2A segment per haploid genome. How many of these segments are transcribed is not known.

The organization of late histone genes

In most organisms, histone genes are present in tens to hundreds of copies per genome and are arranged in clusters (reviewed in ref. 14). The structures of these clusters may be classified in two broad categories; histone and intergenic spacer DNA may be regularly arranged in nearly identical units that are repeated in tandem hundreds of times per genome (for example, in the fly, *Drosophila melanogaster* and the early histone gene repeat of the sea urchin) or they may be irregularly arranged in clusters exhibiting variability in gene orders, polarities of transcription and lengths of intergene spacer DNA (for example, in mouse, chicken, human and the amphibian, *Xenopus laevis*). Recent findings have shown that the distinction between regular and irregular arrangements is not always precise. In the sea urchin, early histone coding sequences have been found in genomic locations remote from the major early histone gene cluster¹³. These histone gene 'orphons' are not organized in clusters but as solitary elements embedded in a DNA context which is completely unlike that of the histone genes in the major cluster.

We have shown here that sea urchin (*S. purpuratus*) late histone genes, like the histone genes of birds and mammals, show variable topology and fine structure. (Similar findings have been obtained recently in the sea urchin, *L. pictus*⁸). Comparison of the four late histone gene clones demonstrates that

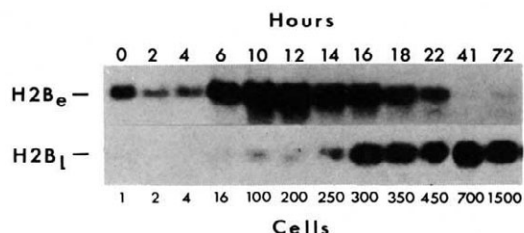
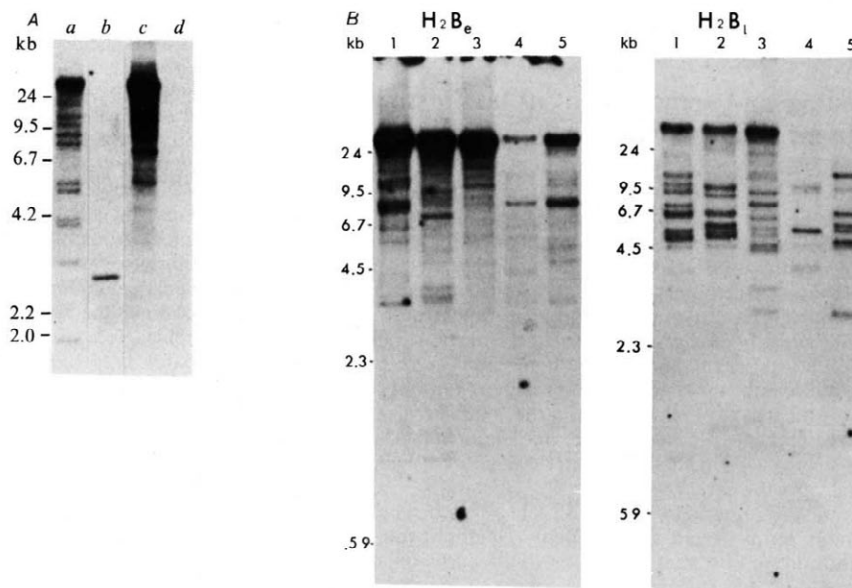


Fig. 3 Developmental profiles of early and late H2B mRNA revealed by hybridization of RNA on gel blots to early or late histone gene probes. RNA was extracted from embryos (obtained from a single mating) at successive stages of development¹⁹. Samples (20 μ g) were treated with glyoxal³³ and electrophoresed in parallel on duplicate 2.2% agarose gels. The RNA was then transferred to DBM (diazobenzoyloxymethyl) paper³⁴ and the blots were incubated in hybridization buffer (50% formamide, $4\times$ SSC, 50 mM sodium phosphate pH 7.0, 0.1% SDS, 250 μ g ml⁻¹ of sheared, denatured calf thymus DNA, and 0.025% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin) containing in addition 1% glycine. The blots were incubated in hybridization buffer containing radiolabelled early or late H2B histone gene fragments (having a specific activity of $\sim 10^7$ d.p.m. per μ g) at a concentration of $\sim 5\times 10^5$ d.p.m. ml⁻¹. Both the early and late histone gene probes included only mRNA coding sequences. The early H2B probe consisted of a 236-nucleotide fragment bounded by *Hae*III sites located at amino acid position 55 and near the 3' terminus of the mRNA. (The H2B protein is 121 amino acids long.) The late H2B probe was a 300-nucleotide *Bam*HI-*Rsa*I fragment extending from position 11 to position 117. Hybridization was carried out at 52 °C for 18 h. Blots were washed in $0.1\times$ SSC, 10 mM sodium phosphate pH 7.0, 0.1% SDS at 42 °C and exposed to preflashed XR-5 film (Kodak) at -80 °C for several days. The images were scanned using a Chromatics densitometer. Control experiments (not shown) demonstrated that the relationship between the quantity of hybridizing RNA on the paper and the intensity of the hybridization signal is linear. The number of hours after fertilization is indicated above the autoradiograms; the number of cells per embryo (derived from the data of Hinegardner³⁵) at the corresponding stages is indicated below. Hybridization of early and late H2B probes is shown in the upper (labelled H2B_e) and lower (labelled H2B_l) panels, respectively.

Fig. 4 Autoradiographs of genomic Southern gel blots showing the number and organization of genomic DNA fragments homologous to early or late histone probes. **A**, *S. purpuratus* DNA from a single individual (4 μ g) was digested with *Bgl*II and electrophoresed on 0.6% agarose gels³⁰. Plasmid pSpL3 (containing late H2A and late H2B coding sequences) was digested with restriction enzymes and electrophoresed in wells adjacent to the genomic DNA. This plasmid was derived from the λ clone L3. The plasmid DNA was added in amounts equivalent to approximately two copies per haploid genome (assuming a *C* value of 0.77 pg, ref. 36). The DNA was blotted onto nitrocellulose paper and the resultant blot was cut into strips, each including a lane of genomic DNA and a lane of plasmid DNA standard. The strips were first preincubated in hybridization buffer (3 $\times$ SSC, 50 mM sodium phosphate, pH 7.0, 0.025% each of bovine serum albumin, Ficoll and polyvinylpyrrolidone) for several hours at 65 °C and then incubated at the same temperature for 18 h in hybridization buffer containing $\sim 10^6$ d.p.m. ml⁻¹ of radiolabelled DNA probe. Blots were washed in 0.1 $\times$ SSC, 0.1% SDS, 10 mM sodium phosphate pH 7.0 at 42 °C for several hours and exposed to X-ray film as described in Fig. 3 legend. All probes contained only mRNA-coding sequences. The late H2A probe consisted of a 211-nucleotide fragment of pSpL3 bounded by *Hinf*I sites at amino acid positions 31 and 98. The early H2A probe (344 nucleotides) included mRNA coding sequence between a *Taq*I site near the 5' terminus of the mRNA and a *Hind*III site at position 94. The early and late H2B probes are described in Fig. 3 legend. Lanes *a* and *b*, late H2A probe hybridized to genomic DNA and to pSpL3 standard, respectively. Lanes *c* and *d*, early H2A probe hybridized to genomic DNA and to pSpL3 standard. **B**, DNA from five individual sea urchins was digested, electrophoresed and blotted as described above. One set of blots was hybridized to early H2B probe and another set was hybridized to late H2B probe. The numbered lanes represent DNA from individuals 1–5 respectively. All other details as described for **A**.



many aspects of their structures differ. These include the identity and proximity of nearest neighbouring histone genes, the relative polarities of transcription of gene pairs, sequences of intergenic spacer DNA, and even the amino acid sequences (derived from DNA sequences) of proteins potentially encoded by the late histone gene sequences. Genomic DNA blotting experiments have shown that late histone genes are present at 5–12 copies per genome, and, like our cloned examples of late histone genes, the others appear to be irregularly arranged. We conclude that early and late histone genes show quite distinct genomic arrangements; thus, in the same organism, members of a multigene family can show both a tandem, regular organization and a dispersed irregular configuration.

Late histone gene expression: how early is 'late'?

Using a late histone gene-specific probe, we have examined the developmental profile of late gene expression. The designation 'late' is a misnomer because we now recognize that these genes are first expressed very early in development, being detectable by the fourth cleavage of the zygote. As development proceeds, there is an increase in the amount of late histone gene transcripts, with a dramatic jump in transcript levels during a short time interval in the late blastula embryo (14–16 h post-fertilization). This increase is in considerable excess over what one might expect merely from the increase in the number of embryonic nuclei during the same period. In addition, the abrupt rise in late gene transcript levels coincides with a marked decrease in the measured rate of early gene transcription¹¹ and a decline in the steady-state level of early mRNAs¹². The switch from early to late histone gene expression cannot therefore result solely from the suppression of early gene activity; it must involve the simultaneous regulation of both early and late histone gene sets. An important and yet unanswered question is whether the expression of these gene sets is spatially segregated in the

embryo or whether their differential regulation is occurring within the same cells.

How late is 'late'? After 18 h of development, the embryonic content of late histone mRNA remains nearly constant, at least until the pluteus larva stage. We have not yet determined the level of late histone gene expression in adult sea urchins but many of the late histone proteins continue to be the major chromatin constituents in adult tissues (L. Cohen, personal communication). Of course, the late histones cannot be the sole adult type. Sperm have been shown to contain an unusual H2B which differs in sequence from both early and late H2Bs¹⁵.

The histone multigene family

What is the significance of different genomic organizations for early and late histone genes? Several explanations seem plausible. The distinct arrangements of the two gene sets may facilitate their differential expression during embryogenesis. Alternatively, the differing early and late gene topologies may result from other selective pressures during the course of echinoderm evolution. The tandem repetition of the early genes may be a consequence of selection for their relatively large numbers compared with the numbers of their late histone gene counterparts. Large numbers of early genes may be required to meet the large demand for histones during the rapid cleavage of the early sea urchin embryo. Tandem duplications of an ancestral early gene cluster could have increased early gene numbers by several orders of magnitude, resulting in the regular repeating structure identified today. Large, tandemly duplicated gene families with repetition frequencies of the order of hundreds or larger are frequently more homogeneous than are less numerous gene families¹⁶. This is to be expected because the frequency of homogenizing cross-over events between sister chromatids should increase with the number of repeated units^{17,18}. Thus, we might expect that any selective pressure for an increase in early gene number should result in early gene

homogeneity if the mechanism of increase is tandem duplication.

Such considerations also suggest a further explanation for histone gene organization. Perhaps the exact homogeneity of early histone gene sequence is important for differential histone gene or protein function. Selection for such homogeneity could have resulted in different early and late gene organizations. Recombinational events associated with tandemly repeated genes could act to maintain uniformity in their nucleotide sequence. Similarly, the irregular arrangement of late histone genes and the consequent low frequency of cross-over events could help maintain the identities of the various, heterogeneous types of late histone proteins.

Evolution of late histone genes

Finally, on the basis of our structural data, we can suggest two alternative models for the evolution of the early and late sets of histone genes. The late gene set may have originated when a recombinational event translocated a segment of the ancestral histone gene cluster to a remote locus in the genome. The recently discovered orphon histone genes¹³ presumably testify to the repeated occurrence of such gene movements. At their

new location, the presumptive late genes would then have acquired their unique developmental pattern of expression and, without the homogenization mechanisms of the tandem family, genetic drift could then have proceeded to bring us to the current situation. Alternatively, the early and late gene sets may be the product of an ancestral divergence between a single gene and its duplicated copy. Differing genomic organizations and repetition frequencies may then have resulted (as discussed earlier) from distinct selective pressures acting on the emerging gene sets.

A phylogenetic survey of histone gene organizations in animals in the echinoderm lineage will be useful in distinguishing between these models. The magnitude of nucleotide sequence differences (our unpublished observations) between early and late histone genes strongly suggests that the two gene sets diverged from one another long ago in the evolution of echinoderms. We would therefore predict that while the temporal patterns of histone gene expression may vary between organisms, early and late histone gene sequences will be widespread among the Echinodermata.

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Two-dimensional crystallization technique for imaging macromolecules, with application to antigen–antibody–complement complexes

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Two-dimensional crystals are formed from macromolecules bound on the surface of a lipid monolayer. A ligand linked to the lipid orientates the binding, and lateral diffusion of the lipids facilitates crystallization. The crystals are suitable for structural analysis by image processing of electron micrographs. An example is the formation of ordered arrays of antibodies on a monolayer of a lipid hapten, and subsequent decoration of these arrays with the first component of complement. Image processing indicates the arrangement of antibodies and the site of complement binding. This approach should be widely applicable to molecular complexes, such as those in replication, protein synthesis, hormone–receptor interaction and metabolic processes.

ORDERED arrays of molecules are required for structure determination by X-ray diffraction or by image processing of electron micrographs. The large, single crystals needed for X-ray studies are often difficult to obtain, and the subsequent analysis is time consuming. Ordering suitable for electron microscopy is rare: it occurs naturally in a few cases, such as two-dimensional arrays of membrane proteins, and can occasionally be induced in special conditions. When the electron

microscopic approach is applicable, however, it is rapid and requires only small amounts of material. The goal of the present studies was to develop a general method of forming two-dimensional crystals and thus to bring a large number of biological macromolecules within reach of structure determination by electron microscopy.

Powerful methods for image processing of electron micrographs have been developed by Klug and his colleagues and

applied to the ordered arrays of proteins found in icosahedral shells, helical fibres and thin crystals^{1,2}. These methods exploit the symmetry of an array to average out the distortions of individual molecules in an electron micrograph and produce an image of only those features that repeat from one molecule to the next. Views of the molecule from different directions, either inherent in the symmetry of the array or obtained by tilting the specimen in the electron microscope, are combined to reconstruct an image of the molecule in three dimensions. Both averaging and three-dimensional reconstruction are accomplished with Fourier transform techniques, in a manner analogous to X-ray crystallographic analysis. Most studies have been done with negatively stained specimens, in which the outlines of molecules are revealed at a resolution of, typically, ~ 25 Å. The results are informative about molecular arrangement and rearrangement, especially when interpreted in conjunction with biochemical and crystallographic data. In the preliminary studies of antigen-antibody-complement complexes reported here, image analysis at ~ 60 Å resolution was indicative of the molecular packing of the antibodies and the nature of the antibody-complement interaction. With novel methods for electron microscopy of unstained specimens, which have given dramatic results in some cases³, it may be possible to extend the resolution of the analysis and obtain internal structural details.

Two-dimensional crystallization technique

Conditions required or favourable for two-dimensional crystallization include: (1) fixation of molecules in a plane, (2) mobility of the molecules within the plane to allow sampling of various bonding arrangements, (3) identical orientation of all the molecules and (4) a high concentration of molecules in the plane, so that crystallization will be favoured over two-dimensional solution. The first requirement is met by the binding of molecules to a surface. However, if the binding is tight enough to ensure a high concentration, the molecules may not hop rapidly from site to site and the requirement for mobility will not be satisfied. This situation occurs in practice when molecules are 'irreversibly' adsorbed on a solid surface. Random binding of the molecules leaves gaps too small to accommodate additional molecules, and because of the lack of mobility, the gaps persist. A maximum surface coverage of 55% is expected for spherical molecules^{4,5} and this coverage is observed in saturation binding of ferritin to a carbon or a Lexan polycarbonate surface⁶.

It occurred to us that these difficulties might be overcome and all the requirements for two-dimensional crystallization met by the use of a lipid monolayer derivatized with a ligand for binding the molecule of interest. Rapid lateral diffusion of the lipids⁶ would ensure the necessary mobility of the bound molecules. The specificity of interaction with the ligand would result in a unique orientation of the bound molecules, and the small size of most lipids, a diameter of ~ 9 Å for a phospholipid⁷, would allow close packing of most types of bound molecule, for example DNA and proteins, typically ≥ 20 Å in diameter.

In practice, the lipid monolayer is deposited on an electron microscope grid in the orientation that exposes the attached ligands. The grid is incubated in a solution of the molecule to be crystallized and is then examined in the electron microscope for ordered arrays. Molecular assemblies may be investigated by transferring the grid to a solution of a second molecule that binds to the first, either before or after an array of the first has formed.

Initially, it was difficult to judge the prospects of obtaining two-dimensional crystals of bound molecules. Although the orientation and high concentration of the molecules are conducive to array formation, the smaller number of intermolecular contacts in two as compared with three dimensions may reduce the extent and degree of order of any crystalline arrays. The study of antigen-antibody complexes was undertaken as an example to determine whether or not two-dimensional crystal-

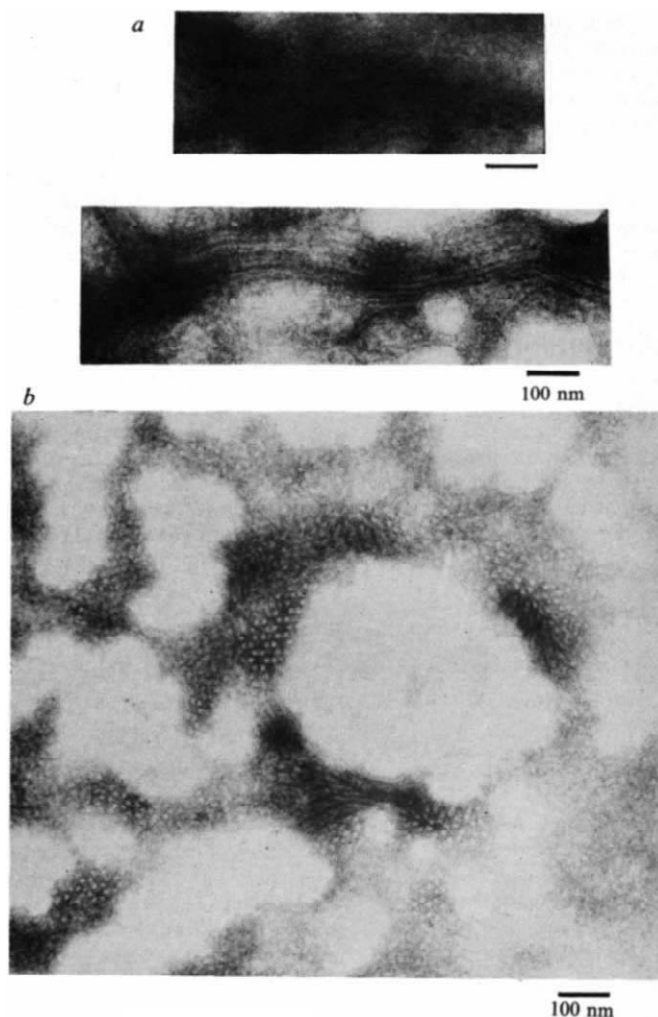


Fig. 1 Electron micrographs of ordered arrays of antibodies on lipid monolayers. Silver grids were used to avoid corrosion problems associated with long exposure to salt solutions. The grids were coated with collodion, carbon and a lipid monolayer, and were floated on drops of antibody solution in microtitre wells at room temperature. After the grids had been withdrawn and washed with several drops of water, they were negatively stained with 1% uranyl acetate. The antibody was purified from ascites fluid by precipitation with ammonium sulphate, chromatography on QAE-Sephadex and gel filtration through Sephadex G-200 as described elsewhere¹⁹. *a*, Upper: linear arrays of anti-DNP antibodies on a DNP-PE monolayer after a 10-h exposure to $250 \mu\text{g ml}^{-1}$ of antibody in 150 mM NaCl, 50 mM Tris pH 8.1. Lower: same, but with a monolayer formed from an equimolar mixture of DNP-PE and PC. *b*, Hexagonal arrays of anti-DNP antibodies on a lipid monolayer as in *a*, lower panel, after a 15-h exposure to $50 \mu\text{g ml}^{-1}$ of antibody.

lization occurs readily, and to establish the principles of the technique.

Two-dimensional crystals of antigen-antibody complexes

A lipid hapten, *N*-dinitrophenyl phosphatidylethanolamine (DNP-PE), was used in conjunction with a monoclonal anti-DNP IgG antibody of the $\gamma 1$ subclass. The formation of closely related lipid hapten-antibody complexes and their lateral mobility in monolayers have been described previously⁸. In that work, lipid monolayers were transferred from a water surface

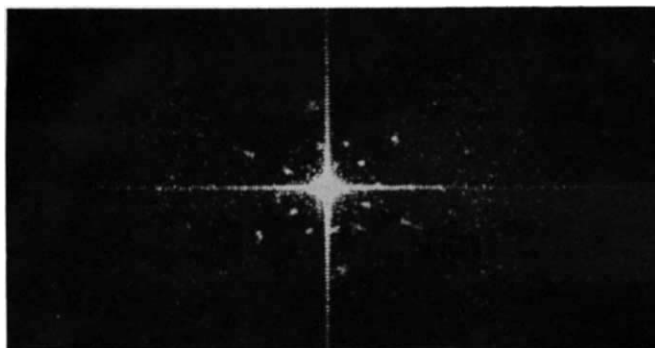


Fig. 2 Optical diffraction pattern from a hexagonal array of antibodies as in Fig. 1b.

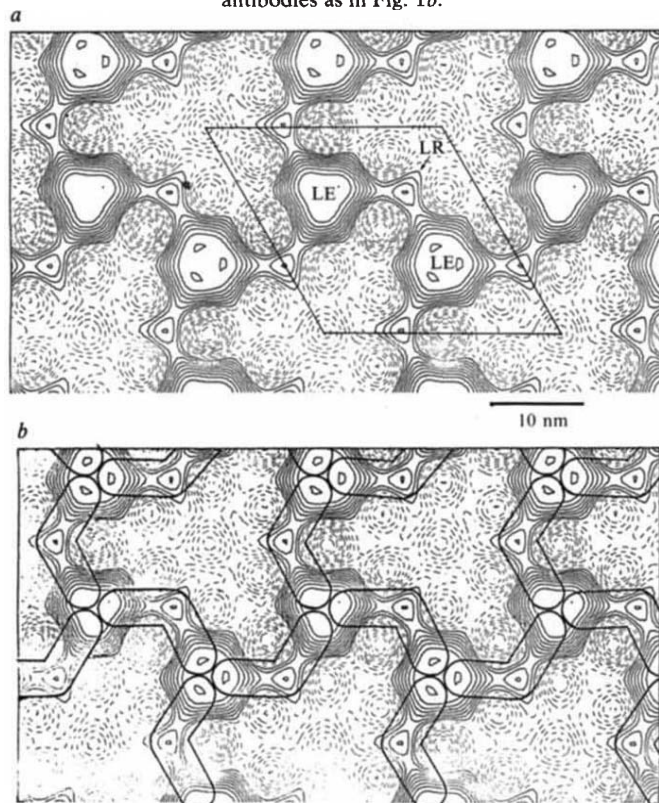


Fig. 3 *a*, Projected structure of a hexagonal array of antibodies. The outlines of a unit cell are drawn, and the centres of the large stain-excluding elements (LE) and linking region (LR) are indicated. An image as in Fig. 1b was processed as described in the text. Amplitudes and phases from five sets of 3-fold related reciprocal lattice points, extending to the 2,2 set, were used. Two additional images were analysed and the results were similar to those shown. In particular, the location and appearance of the linking regions were essentially the same. An image was also analysed without averaging the 3-fold related data; again, similar results were obtained, although with some distortions. *b*, Proposed arrangement of antibodies in a hexagonal array. A highly schematic drawing of an antibody array is superimposed on the projected structure in *a*.

by a version of the Langmuir-Blodgett technique, and a similar procedure was used here. A mixed monolayer of DNP-PE and phosphatidylcholine (PC) was spread on an air-water interface in a Teflon trough by the application of a few drops of the lipids in hexane solution. An electron microscope grid, freshly coated with carbon, was passed through the interface, resulting in the deposition of a lipid monolayer with the hydrocarbon chains abutting the hydrophobic carbon surface and the polar head groups facing the aqueous solution. The surface of the trough was swept free of lipid, and the grid was withdrawn into air and transferred to a drop of antibody solution. When the

grid was examined immediately by negative staining with uranyl acetate and electron microscopy, the surface appeared densely covered with protein. When grids were exposed to antibody for some time before staining and microscopy, two types of regular pattern were observed. The first, a linear pattern (Fig. 1a), was seen after 1–3 h incubation at high concentrations of antibody. Both the linear pattern and a second, hexagonal one were obtained from more dilute solutions after 8–18 h.

The formation of linear and hexagonal patterns required DNP-PE monolayers and anti-DNP antibody, indicating that the patterns actually contain the antibody. No patterns, or even significant adsorption of protein, were detected with PC monolayers and anti-DNP antibody, or with DNP-PE and a nonspecific antibody, such as rabbit IgG or a monoclonal anti-dansyl IgG. Nor was there any binding of bovine serum albumin to PC or DNP-PE monolayers in the conditions of pH and ionic strength used. Nonspecific adsorption and hexagonal ordering did occur with ferritin, as first noted by Fromherz⁹. However, hexagonal patches of ferritin also form on a carbon surface in the absence of a lipid monolayer⁵. Apparently, due to the symmetrical shape of the ferritin molecule and to strong intermolecular interactions, ordered arrays can form without a mobile surface or oriented binding.

Whereas the linear and hexagonal patterns in Fig. 1 were obtained only when lipid and antibody of appropriate specificity were used, there was no strong dependence on solution conditions. It did not prove necessary to induce crystallization by adding precipitants or by systematic adjustment of other variables. Most experiments were carried out in nearly physiological conditions of pH and ionic strength, where the antibody was fully soluble; similar results were obtained at other pH values in the range 7–9. The comparative ease of ordering antibodies is probably due to the high concentration and orientation of the bound molecules.

Molecular packing in linear and hexagonal arrays

The linear patterns in Fig. 1a are characterized by stain-excluding stripes with a lateral spacing, or centre-to-centre distance, of 150 ± 10 Å. Each stripe is itself divided down the middle by a thin line of stain, and in some electron micrographs (Fig. 1a, lower) the stripes show transverse lines, or striations, with a spacing of ~ 40 Å. The linear patterns are readily interpreted in terms of the packing of IgG molecules with both Fab arms bound to the lipid monolayer and the Fc tail projecting into solution: the stripes correspond to rows of molecules stacked with Fab-Fc-Fab planes flat against one another; the striations correspond to individual antibody molecules viewed in projection down their Fc tails. The spacings of the stripes and striations are in good agreement with the corresponding molecular dimensions of 142 and 50 Å obtained from X-ray crystallography¹⁰. The line of stain down the middle of a stripe is initially surprising, as exclusion of stain by the Fc tail might be expected in this region. The likely explanation is that stain fills the space between the Fab arms in the inverted 'Y' configuration of the antibody on the grid, obscuring any effect of the Fc tail directly above. Similar lack of stain exclusion by the Fc region is seen in electron micrographs of antibody crystal faces¹¹.

The degree of order in these linear arrays of antibodies was not high. Optical diffraction patterns showed peaks corresponding to only first and second orders of the spacing in the direction transverse to the stripes and no diffracted intensity in the direction along the stripes. In contrast, the hexagonal ordering seen in Fig. 1b was truly crystalline. The ordered regions are characterized by a hexagonal pattern of large stain-excluding elements with a spacing (centre-to-centre distance) of 150 ± 10 Å. The extent of the ordered regions was not large, but the degree of order within a region was high. Optical diffraction from such regions gave patterns (Fig. 2) that could be indexed on a hexagonal lattice. The diffraction extended to at least four orders, corresponding to a resolution of ~ 60 Å.

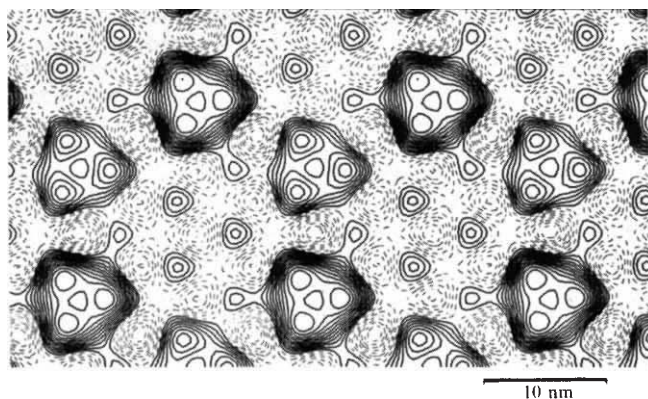


Fig. 4 Projected structure of an antibody lattice decorated with C1q. A grid was exposed to $50 \mu\text{g ml}^{-1}$ of antibody for 8 h and then to $50 \mu\text{g ml}^{-1}$ of C1q for 10 h as in Fig. 1a, upper panel. Image processing was as in Fig. 3a. The difference in stain exclusion between adjacent large elements was a consistent feature (6 out of 18 levels in the example shown here and 9 out of 18 levels in a second processed image), whereas the minor peaks were not reproducible.

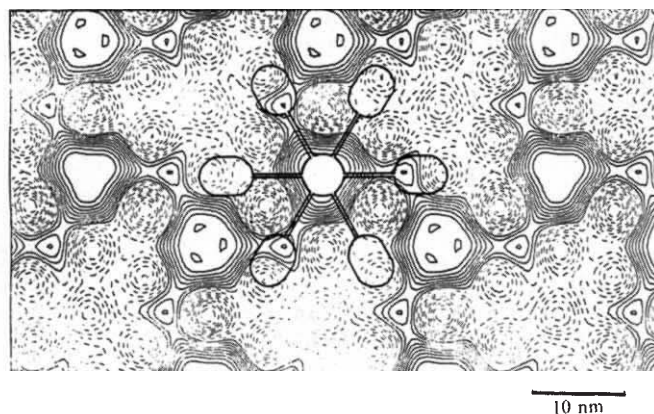


Fig. 5 Proposed location of C1q on an antibody lattice. A highly schematic drawing of C1q, with dimensions from previous electron micrographs¹⁶, is superimposed on the projected structure from Fig. 3a.

For further analysis of the hexagonal patterns, standard methods of image processing were used^{2,12,13}. Optical diffraction was used to select an area of a micrograph having a high degree of order. This area was analysed by densitometry with a step size corresponding to 8 Å, and the Fourier transform of the optical densities was computed. The peaks in the transform were indexed on a hexagonal reciprocal lattice corresponding to unit cell dimensions of 260×260 Å. A unit cell of this size contains two of the stain-excluding elements spaced 150 Å apart in a hexagonal system with p3 plane group symmetry. The origin of the transform was refined by minimizing the differences in phase between 3-fold related peaks. In the example shown (Fig. 3a), a root mean square (r.m.s.) phase deviation for five sets of peaks of only 15° was obtained, indicating good conformity with and regularity of the plane group lattice. The amplitudes and phases of symmetry-related peaks were averaged and the resulting values were recombined by Fourier synthesis to give a 'noise-filtered', symmetry-averaged image.

A notable feature of the processed image (Fig. 3a) is the presence of weakly stain-excluding regions linking the large stain-excluding elements. These linking regions appear bent: lines to the centres of the adjacent large elements intersect at an angle of $\sim 120^\circ$. The bend defines a sense of rotation about the 3-fold axis at the centre of a large element, and this sense alternates from one element to the next. The alternation accounts for the occurrence of two large elements in the unit cell, with an apparent mirror plane through the linking region between them. The plane group p3 does not require the two large elements to be equivalent, although they appeared virtually identical in every example analysed.

The 150-Å spacing of large elements and of linking regions is similar to the 150-Å span of antibodies in linear arrays and three-dimensional crystals. This suggests the two alternative arrangements in which antibody molecules extend from one large element to the next or from one linking region to the next. In the first arrangement (Fig. 3b), Fab arms are trimer-clustered about the 3-fold axis in the centre of a large element and Fc tails lie in the linking regions. In the second arrangement (not shown) the situation is reversed, with Fc tails in the large element and Fab arms in the linking region. We favour the first arrangement because it assigns Fc tails to weakly stain-excluding regions, and a similar lack of stain exclusion was noted for Fc tails in linear arrays and three-dimensional crystals (see above). The strength of the interpretation is that a single set of molecular dimensions and a single pattern of negative staining can account for the appearance of antibodies in linear, hexagonal and three-dimensional crystalline arrays.

The individual Fab arms in trimer clusters could not be distinguished at the resolution of this analysis. The schematic

representation of antibodies in Fig. 3b was drawn with a 120° angle between Fab arms to fit the bend of the linking region, but other angles would be obtained with different shapes of the arms. As the antibody is viewed in projection, a bent shape implies that the molecule does not stand perpendicular to the plane of the electron microscope grid, but is slightly tilted.

Two-dimensional crystals of antigen-antibody-complement complexes

The association of antibody with antigen and subsequent binding of the C1q component of complement to the Fc tail initiates the 'classical' complement pathway^{14,15}. In the case of IgG, two or more antibody molecules in close proximity are believed to be required for complement activation. The closely packed two-dimensional arrays of antibody-antigen complexes described above, with Fc tails exposed on the surface, seemed ideally suited for interaction with complement. This provided an opportunity both for structural studies on the antigen-antibody-complement complex and for testing the proposed molecular packing in the hexagonal array shown schematically in Fig. 3b. We expected that decoration of the antibody lattice with C1q would result in increased stain exclusion by the Fc tails in the linking regions of the array.

For C1q decoration, a DNP-PE-coated grid was exposed to antibody for 8 h and then to C1q for a further 10 h. The resulting electron micrographs showed both linear and hexagonal arrays. The regions of hexagonal ordering were similar in size and quality to those found with antibody alone, but on image processing, a significant difference emerged. The antibody lattice decorated with C1q showed a striking alternation in stain exclusion from one large element of the array to the next. The difference between adjacent elements was 6 contour levels out of 18 in the example shown (Fig. 4). In contrast, with antibody alone, the difference was never greater than 1 level out of 18. This effect of C1q on the staining of large elements of the antibody array was surprising both in its alternation and because we had expected a more pronounced effect on the linking regions of the array.

One of many conceivable explanations of these results follows from the proposed molecular packing of antibodies (Fig. 3b) and the structure of the C1q molecule. Electron microscopic and biochemical studies have led to a model for C1q with six thin, collagenous fibres radiating from a central, globular region and terminating in Fc-binding heads¹⁶⁻¹⁸. Due to the flexibility of the collagenous fibres, the maximum dimension (head-to-head distance) of the molecule varies, ranging in electron micrographs over 270–380 Å. If a molecule at the lower end of this size range is centred on a large element of an antibody array (Fig. 5), three Fc-binding heads coincide in position with linking regions of the array, the proposed location of antibody Fc tails. Because the linking regions thus occupied are shared with

neighbouring large elements, additional C1q molecules cannot be placed on these elements, but only on the next nearest ones. This mode of C1q binding accounts for the alternation in stain exclusion seen in the electron microscope (Fig. 4). It also has the advantage of associating the apparent 6-fold axis of the C1q molecule with a 3-fold axis of the antibody lattice. This results in the most efficient coverage of the lattice, with the minimum number of C1q molecules and maximum number of bonds per molecule, and thus the minimum free energy of antibody-complement interaction.

It may seem that limiting coverage of an antibody lattice will be reached when either one or two large elements remain free between sites of bound C1q. The image processing results (Fig. 4), however, indicate that free elements occur singly, interspersed with bound C1q in alternating fashion. Possible explanations for this include a difference in affinity of the two large elements in the unit cell for C1q, or reversible binding and consequent saturation with C1q at the high concentrations used in these experiments.

Prospects

The ease of forming two-dimensional crystals of antibodies and their suitability for image analysis may be attributed to the high concentration and orientation of molecules bound on a lipid monolayer and to the possibility of analysing even the smallest crystals in the electron microscope. For these reasons, the two-dimensional crystallization technique may be generally applicable to molecules that are, like antibodies, difficult to crystallize in three dimensions. The range of the technique is further extended by the sequential addition of components to form molecular assemblies, exemplified here by antigen-anti-

body-complement complexes. In future studies it should be possible to form and crystallize such complexes as those in replication, protein synthesis, hormone-receptor interaction and metabolic processes. As crystallization occurs in physiological conditions, it may even be possible to add substrates, elicit the functions of these complexes and obtain images of reaction intermediates and products.

Whereas the formation of monolayer crystals of antigen-antibody-complement complexes is demonstrated here, the structural analysis of the crystals should be regarded as preliminary. Many molecular packing arrangements are consistent with the data, and further information is needed to decide among them. If the proposed arrangements (Figs 3b, 5) prove correct, some aspects may be significant. The mode of antibody-C1q interaction may have physiological relevance. DNP-PE in a lipid monolayer is similar in many respects to cell-surface antigens, and antibodies associated with these antigens may form trimer clusters or lattices that facilitate C1q binding and complement fixation. Whatever the relevance of antibody lattice formation, the site of C1q binding on the lattice is of interest. Binding on a symmetry axis provides evidence for the multiple antibody-C1q interactions believed to initiate the complement pathway. Further image analysis at higher resolution and in three dimensions should reveal the precise site on the Fc tail that serves as the C1q receptor.

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Targeted integration of baboon endogenous virus in the *BEVI* locus on human chromosome 6

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The infection of cultured human cells with baboon endogenous virus (BEV) frequently leads to an association of viral DNA with a specific genetic locus (termed BEVI, for baboon endogenous virus infection) on chromosome 6. Restriction endonuclease digestion of DNA from BEV-infected human cells and their derived somatic cell clones frequently revealed a common cellular DNA sequence in the proximity of one of the junctions between cellular DNA and the integrated virus. We propose that a short cellular DNA sequence, repeated on chromosome 6 and separated by unique DNA sequences, presents a high-affinity target for the integration of BEV in human cells.

INFECTION of mammalian cells with competent retroviruses results in the reverse transcription of genomic RNA into DNA that integrates into the chromosomal DNA of the host cell. The results of molecular analyses in several systems¹⁻¹⁶ indicate

that viral DNA integration is sequence specific with respect to the viral genome but nonspecific (or even random) with respect to the host genome. These conclusions are supported both by restriction endonuclease studies of proviral sequences¹⁻¹⁶ and

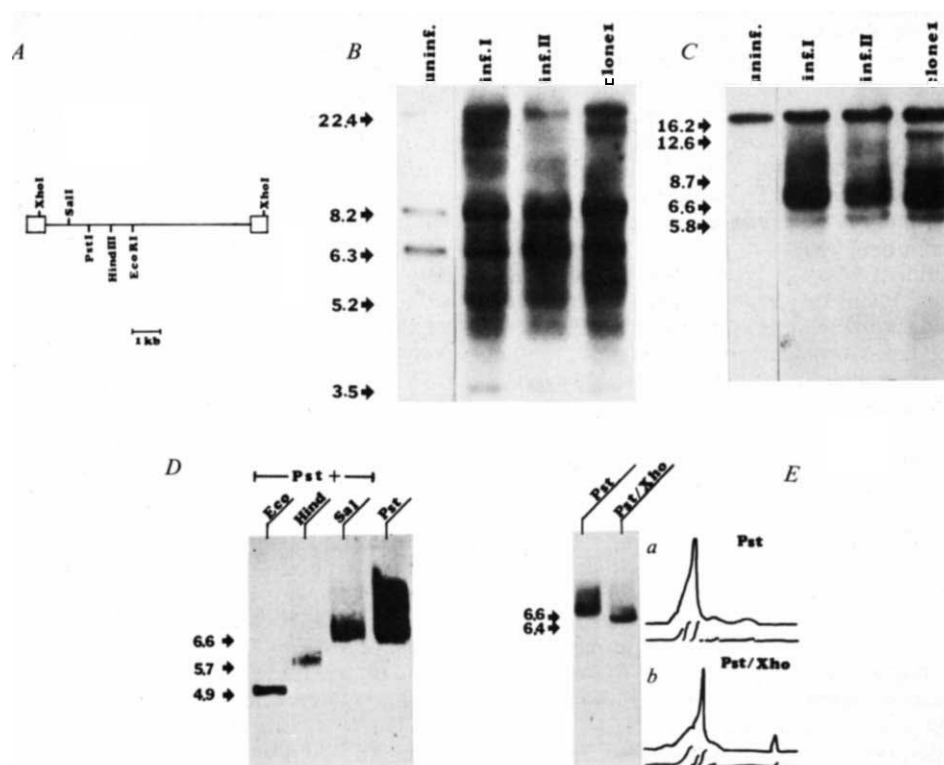


Fig. 1 Restriction endonuclease studies of DNA from BEV (M7 strain) infected human cells. Uninfected VA-2 cells (uninf.), and either cloned or high and low multiplicity of infection (inf.I and inf.II, respectively) mass cultures of BEV-infected VA-2 cells were treated with proteinase K (100 g ml^{-1}) and SDS (1%) in DNA extraction buffer (0.02 M Tris-HCl, pH 8.1, 0.01 M EDTA, and 0.1 M NaCl). DNA was subsequently extracted using phenol-chloroform (2:1) and concentrated by spooling on a glass rod in the presence of ethanol. DNA was resuspended in 0.005 M Tris-HCl, pH 7.4, and 0.0001 M EDTA and quantitated by UV absorption. DNA was digested with various restriction endonucleases including *EcoRI*, *PstI*, *HindIII*, *SalI* and *XhoI* in conditions recommended by the supplier (New England Biolabs). On completion (as measured by a plasmid control), digested DNA ($10 \mu\text{g}$) was electrophoresed on a 0.8% agarose gel in 0.02 M Tris-acetate, pH 8.15, 0.009 M NaCl and 0.001 M EDTA. Radiolabelled *HindIII*-digested λ bacteriophage DNA was included as a molecular weight marker in all gels. After electrophoresis, DNA was transferred to nitrocellulose sheets by the method of Southern²⁸. Virus-specific bands were detected by hybridization with ^{32}P -labelled complementary DNA synthesized from a cloned unintegrated BEV DNA in pBR322 using calf thymus oligomers as primers in a reverse transcriptase-catalysed polymerization. Filters were hybridized for 48–72 h with cDNA followed by extensive rinses to remove unhybridized cDNA as described previously². Fragments were visualized by autoradiography on Kodak XAR-5 film. The size (kb) of specific fragments is indicated on each gel. **A**, Restriction endonuclease map of BEV DNA obtained by Cohen *et al.*²⁹. **B**, *EcoRI* digestion of DNA from uninfected and infected VA-2 cells. **C**, *PstI* digestion of DNA from uninfected and infected VA-2 cells. **D**, Double digestion of DNA from VA-2 cells infected with BEV at a high MOI. **E**, Quantitation of BEV-specific DNA fragments was accomplished by scanning on a Joyce-Loebl densitometer with a microcomputer-based program (Visichart, Interactive Microware, Inc.) for area integration. *PstI* (*a*) and *PstI*-*XhoI* (*b*) digestions were performed in triplicate and the average of three independent scans was used to obtain the integrals for each peak. To illustrate the limited area integrated in the 6.6-kb *PstI* and the 6.4-kb *PstI*-*XhoI* fragments, the lower tracings in both *a* and *b* represent the total integrals distinguishable in the densitometer scan (upper tracing).

by direct sequencing of the virus-host cell junction fragments^{17–19}. Although these data are consistent with nonspecific integration, they may indicate only that the integration sites on chromosomal DNA are multiple^{20–22}, as mapping procedures are inadequate to determine whether a repeated host sequence is common to integration sites used by these retroviruses^{18,20,21}. Several authors have speculated that the ordered, directed nature of retroviral integration with respect to viral sequences might reflect the existence of a short specific integration sequence that occurs in many hundreds of copies in the host cell DNA^{22,23}.

The *BEVI* (for baboon endogenous virus infection) locus has been described previously on human chromosome 6 and is required for infection of rodent-human somatic cell hybrids with baboon endogenous virus (BEV)²⁴. Molecular hybridization analysis of somatic cell hybrids infected with BEV demonstrated that integrated proviral DNA sequences were concordant with the presence of human chromosome 6 and discordant with that of other human chromosomes²⁵. These results prompted the suggestion that *BEVI* is either a preferred or high-affinity integration site of BEV in the human genome.

The finding that chromosome 6 is required for virus replication was confirmed by Brown *et al.*²⁶, who detected not only the concordance with chromosome 6, but also concordance of BEV infectivity with human chromosome 19. This gene on chromosome 19 may encode a receptor for BEV, as Schnitzner *et al.*²⁷ have also placed a receptor gene for the BEV-related virus, feline RD-114, on chromosome 19.

Association of BEV proviruses with a specific human sequence

To study the molecular aspects of integration of BEV in human cells, DNA from human VA-2 cells infected with BEV (M7 strain) at low and high multiplicities of infection was digested with restriction endonucleases (*EcoRI* and *PstI*), fragments were separated on agarose gel electrophoresis, and virus-specific fragments were detected after transfer to nitrocellulose sheets²⁸ by hybridization with radiolabelled, BEV-specific cDNA and autoradiography. DNA from uninfected VA-2 cells was included as a control for BEV-related human sequences not acquired by infection. Finally, DNA from the original cell

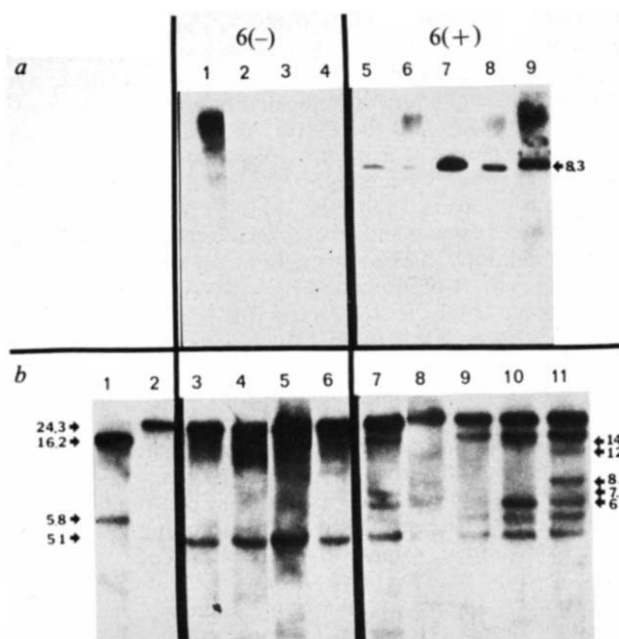


Fig. 2 Segregation of BEV DNA in human-hamster hybrid cells. DNA from either previously described²⁵ somatic cell hybrids formed between clone 1 (see Fig. 1a, b) BEV-infected VA-2 cells and BHK-B1 hamster cells or uninfected VA-2 and BHK-B1 cells (b, lanes 1, 2) was digested with either *XhoI* (a) or *PstI* (b) and virus-specific fragments detected as described in Fig. 1 legend.

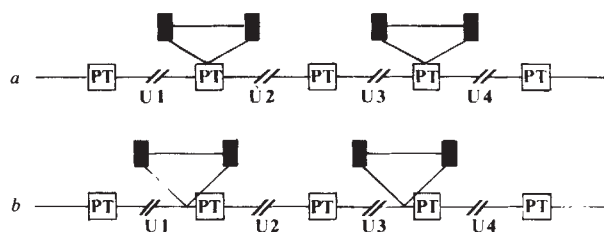


Fig. 3 Proposed model for the *BEVI* locus based on available genetic and molecular data. The *PstI* target sequence is represented by the blocks labelled PT. Unique sequences are indicated by U1-U4. The BEV provirus (■---■) is shown integrating either into (a) or adjacent to (b) the target sequence.

clone (clone 1) used in the formation of human-hamster cell hybrids^{24,25} was used, although this clone was passaged extensively before DNA extraction. For reference with the enzymes used here, we present the restriction endonuclease map of linear BEV DNA (Fig. 1a) obtained by Cohen *et al.*²⁹ and confirmed in our laboratory (data not shown).

EcoRI digestion of DNA from uninfected human cells (Fig. 1b) yielded three BEV-related fragments (22.4, 8.2 and 6.3 kilobases (kb)). This 8.2-kb fragment seems to be similar to that described by Bonner, O'Connell and Cohen³⁰. However, DNA from each of the infected cells produced a random pattern of hybridization for BEV-specific sequences that were unique to infected cultures. Unique fragments are seen in the cloned, infected cell line; however, the random hybridization is clear evidence of re-infection during the extensive passage of these cells. These data suggest the presence of multiple sites for BEV integration in the human chromosome, similar to those obtained with other retroviruses¹⁻¹⁶.

The apparent chromosome specificity of the *BEVI* locus revealed by somatic cell genetics was not easily resolved in light of the apparent lack of host sequence specificity observed in

this initial restriction endonuclease digestion. Two minor, virus-specific fragments (5.2 and 3.5 kb) are common to all the *EcoRI* digestions of BEV-infected cells, suggesting that some preferred integration events occurred, but not all, as suggested by genetic analyses²⁵. Furthermore, digestions with other restriction endonucleases (*HindIII* and *XbaI*; data not shown) produced results similar to those obtained with *EcoRI*. However, digestion of infected cell DNA with *PstI* (Fig. 1c) yielded a major, BEV-specific fragment from all independently infected cells. DNA from uninfected cells produced two virus-related fragments (16.2 and 5.8 kb) after *PstI* digestion. Infected cell DNA, in addition to these fragments, consistently produced a BEV-specific fragment 6.6 kb in size. Because there is only one *PstI* site in BEV DNA (see Fig. 1A, also ref. 30 and unpublished observation) finding the ubiquitous 6.6-kb fragment suggested that a common *PstI* site occurred in cellular DNA either at or adjacent to the virus-host DNA junction of many BEV proviruses. In addition, the novel 6.6-kb fragment was observed in other BEV-infected human cell lines (WIDR and Detroit 562; data not shown), indicating that this fragment was not unique to the VA-2 cell line.

The origin of the novel 6.6-kb *PstI* fragment would depend on the orientation of the integrated provirus and the specificity of the integration event. The BEV DNA restriction endonuclease map (Fig. 1A) shows that *PstI* cleaves at a single site in unintegrated DNA, resulting in two fragments of 2.3 and 6.5 kb representing the 5' and 3' ends of the genome, respectively. *SalI* cleaves at a single site on the 5' side of the *PstI* site, whereas both *EcoRI* and *HindIII* cleave on the 3' side. Therefore, a series of double digestions of infected cell DNA with *PstI* and *SalI*, *HindIII* or *EcoRI* were performed to determine the derivation of the 6.6-kb *PstI* fragment common to all BEV-infected human cell DNAs. The hybridization of these filters was performed using a cDNA probe synthesized from a clone of unintegrated BEV DNA. In the conditions used in this experiment, this cDNA does not resolve the human endogenous sequences, thus facilitating the characterization of the BEV proviruses in infected cells. The results of the double digestions of acutely infected VA-2 cells are given in Fig. 1D.

The mobility of the ubiquitous 6.6-kb *PstI* fragment was not altered by subsequent digestion with *SalI*, but was diminished by treatment with either *EcoRI* or *HindIII*. These data indicate that the novel 6.6-kb *PstI* fragment must be derived from the 3' end of BEV proviruses and that the BEV provirus integrated in only one orientation to, and at an invariable distance from, a specific host cell sequence which included a *PstI* site.

The extent of involvement of the site-specific *PstI* site in human DNA with multiple BEV integrations can be estimated by comparing the intensity of the novel 6.6-kb *PstI* fragment with that of a 6.4-kb *PstI*-*XhoI* double-digestion internal fragment (see Fig. 1A), because the *PstI*-*XhoI* fragment would be derived from all proviruses and contain about the same mass of virus-specific sequences as the novel *PstI* virus-host cell junction-containing fragment. The results of such an analysis (Fig. 1E) revealed that these two fragments have nearly identical intensities. Densitometer scanning of multiple autoradiograms and repeated computer-assisted integration of the 6.6-kb *PstI* (panel a) and the 6.4-kb *PstI*-*XhoI* (panel b) fragment peaks (lower tracings indicate limits of area integrated in each peak) revealed an average of 1.08 for the ratio of intensities of the two fragments. Although the presence of multiple minor bands co-migrating with these fragments could affect the accuracy of this determination, one could conclude that a large proportion of BEV integrations in human cells occurred adjacent to a cellular *PstI* site.

Concordance of proviral integration with human chromosome 6

Human-hamster hybrid clones both positive and negative for human chromosome 6 were analysed for BEV sequences to confirm the association of BEV proviruses with chromosome 6. The enzyme *XhoI*, which cleaves once in each of the long

terminal repeats (LTRs) of BEV (Fig. 1A), generated a single BEV-specific fragment from DNA of only infected somatic cell hybrids that were positive for human chromosome 6 (Fig. 2a, lanes 5–9). Chromosome 6-negative clones (lanes 1–4) do not exhibit the expected 8.3-kb fragment. In addition to confirming the association of BEV DNA with chromosome 6, this observation indicates that the BEV provirus is integrated in a co-linear orientation to that observed with unintegrated viral DNA (shown schematically in Fig. 1A) and that viral DNA recombination with host cell DNA occurs at the termini of the LTRs^{1,17}. Thus, these data indicate not only that BEV integrations are restricted to the *BEVI* locus on chromosome 6 but also that the 6.6-kb fragment is not generated by some unusual permutation of BEV DNA.

*Pst*I digestions of DNA from the human-hamster somatic hybrids (Fig. 2b) were performed to establish that the site-specific integration included in the novel 6.6-kb *Pst*I fragment segregated with the *BEVI* locus on chromosome 6. As with *Xho*I digestions, only the BEV-related sequences seen in the uninfected parental DNA (lanes 1, 2) were observed in chromosome 6-negative clones (lanes 3–6). In chromosome 6-positive clones (lanes 7–11), the novel 6.6-kb *Pst*I fragment representing the 3' end of BEV proviruses was always present. (The variable intensities of these fragments in each clone is due to the percentage of cells that are positive for chromosome 6, because after cloning, the human chromosomes continue to segregate in the somatic cell hybrids.) In addition, other BEV-specific fragments (14.2, 12.6, 8.7 and 7.8 kb) derived from the 5' end of the provirus were observed. Because there is no common *Pst*I site on the 5'-flanking cellular sequences, the size of these fragments varies among the clones.

Conclusions

Results presented by Lemons *et al.*^{24,25} demonstrated that a locus, *BEVI*, on human chromosome 6 was required for BEV replication in human cells and somatic hybrids, for maintenance of integrated proviruses in hybrids dynamically segregating human chromosomes, and for re-infection of hybrids that lose integrated proviruses due to chromosome loss. Data presented here confirm the specific association of BEV proviruses with chromosome 6 and indicate the probable sequence-specific integration of this virus in the human genome. Digestions of infected human cell DNA with *Eco*RI (Fig. 1B) and other endonucleases (*Xba*I and *Hind*III; data not shown) produced a heterogeneous pattern of virus-specific fragments characteristic of random integration. However, digestion of these DNAs with *Pst*I generated a novel 6.6-kb fragment that was present as a conspicuous, ubiquitous fragment in mass-infected cultures of human cells. The 6.6-kb *Pst*I fragments seem to be derived from most BEV integration events and generated by a *Pst*I site

located at or near the 3' virus-host cell junction. These data suggest that the *BEVI* locus represents a series of sequence-specific integration sites defined by *Pst*I digestions interspersed among unique sequences, as indicated by the random pattern produced by *Eco*RI and the *Pst*I 5' end fragments. Although this finding is unusual for a retrovirus, a consensus target site sequence has recently been reported for the preferred integration of the transposon *Tn10* (ref. 31) in prokaryotes.

Two possible caveats must be considered in relation to this interpretation. First, a *Pst*I site might be present in the 3' LTR and not the 5' LTR of integrated BEV DNA. However, asymmetry between the LTRs of a retrovirus has not been seen in any of the viruses that have been completely sequenced, nor was asymmetry of LTRs for *Pst*I sites observed in cloned BEV DNA. Furthermore, the 6.6-kb fragment is too large to account for the loss of viral sequences required for an asymmetrical site in a LTR. The second possibility is that a *Pst*I site is generated by the recombination between virus and human sequences and their junction. The virus could theoretically contribute as many as five of the six bases of a *Pst*I site, with only the final base derived from human sequences. This occurrence would not indicate specific sequence recognition by BEV during integration. However, if one examines the sequence of the LTR obtained by Tamura, Noda and Takano³², the terminal nucleotides for the M7 strain of BEV are 5'-AAATGAAAAGTAA-/-TGATTCTAACATC-3'. As the recognition sequence for *Pst*I is 5'-CTGCAG-3', only a single base could be contributed from the virus terminus, suggesting that the host recognition sequence must be a minimum of five bases. If, as suggested by some authors, the two terminal nucleotides are deleted during integration of retroviruses¹⁷, the complete *Pst*I site would be of cellular origin.

From the combined results of genetic and molecular data we postulate the model presented in Fig. 3. Most integrations of BEV DNA are associated with a *Pst*I-defined 'target sequence' (PT). Viral DNA may be inserted either directly into (Fig. 3a) or immediately adjacent to (Fig. 3b) the target sequence. This human sequence recognized by BEV DNA is apparently repeated and separated by unique cellular sequences (U1, U2, U3, etc.) that are visualized by the 3'- and 5'-flanking DNA heterogeneity generated by enzymes other than *Pst*I and the 5' end in the *Pst*I digestion. The number of these target sequences, their length, spacing on chromosome 6 and appearance on chromosomes other than chromosome 6 are unknown.

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Facular influences on the apparent solar shape

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The apparent shape of the Sun is controversial¹: Dicke and Goldenberg² measured a Sun with an oblateness (equatorial minus polar radius divided by polar radius) of 5×10^{-5} , whereas Hill and Stebbins measured a value of 0.9×10^{-5} , consistent with a slowly rotating core. More recently, Claverie *et al.*³ have found the Sun's interior may be rotating faster than the surface after all. Dicke and Goldenberg's observations were questioned by Chapman and Ingersoll⁴ on the basis that facular influences could introduce a signal which appeared similar to the solar oblateness signal both in shape and magnitude. Dicke⁵ analysed the data in many different ways (such as dividing the data on the basis of facular brightness, and different window size) and concluded that the faculae did not contribute significantly. We reexamine this question by means of more general facular contrast models. We find that the faculae can contribute a signal which has a time dependence similar to the Dicke and Goldenberg oblateness signal, and which for a facular contrast within the range of acceptable values allows an acceptable fit to the oblateness measurements.

The facular contribution to the light intensity can quite generally be represented by the formula⁶:

$$I_{\text{obs}} - I_p = I_p \sum A_i \mu [a_p + b_p \mu + c_p \mu^2] F_c(\mu) \quad (1)$$

where I_{obs} is the observed specific intensity in an active region, I_p is the same quantity for the undisturbed photosphere, A_i is the facular area, F_c is the facular contrast, $\mu = \cos \tau$ (τ is the angular distance of the active region from the apparent centre of the solar disk). The bracketed term represents standard photospheric limb darkening. For our model of facular contrast⁶, $F_c(\mu)$ is given by the formula:

$$F_c(\mu) = a_i + b_i \mu + c_i \mu^2 - 1 \quad (2)$$

where the coefficients are:

$$a_p = 0.2558, b_p = 0.9732, c_p = -0.2289, a_i = 1.205, \\ b_i = -0.4, \text{ and } c_i = 0.2.$$

In the work of Chapman⁷, on the other hand, a facular contrast was chosen as:

$$F_c(\mu) = 0.04(1/\mu - 1) \quad (3)$$

Both expressions fit the solar observational data⁸.

Here we have calculated the 'diagonal components of the oblateness signal from the faculae observed on the Sun using Caplage data published by NOAA in their solar geophysical data bulletin, for both contrasts. We use a 12.8 arc s exposed limb signal modelled to the Dicke and Goldenberg type experiment (the second harmonic of the signal). Our findings from the Sofia *et al.* model give a value near 10^{-6} for the maximum facular influence, which is consistent with Dicke and Goldenberg. However, when we used the Chapman⁷ faculae limb contrast, a much larger facular effect results, in agreement with the findings of Chapman and Ingersoll⁴. The origin of this behaviour may be understood by examining Fig. 1. The left side of Fig. 1 shows the contrast for the Chapman faculae models^{7,9} increasing as $1/\mu$ compared with our model II (equation (2), curve II)⁶ where the contrast is only parabolic in μ . The observations of Frazier⁸ are also shown with uncertainties. Although any of these representations is adequate for $0.2 < \mu \leq 1.0$ (as shown on the left), for $0.0 < \mu \leq 0.2$, the contrast multiplied by μ (shown on the right), begins to differ

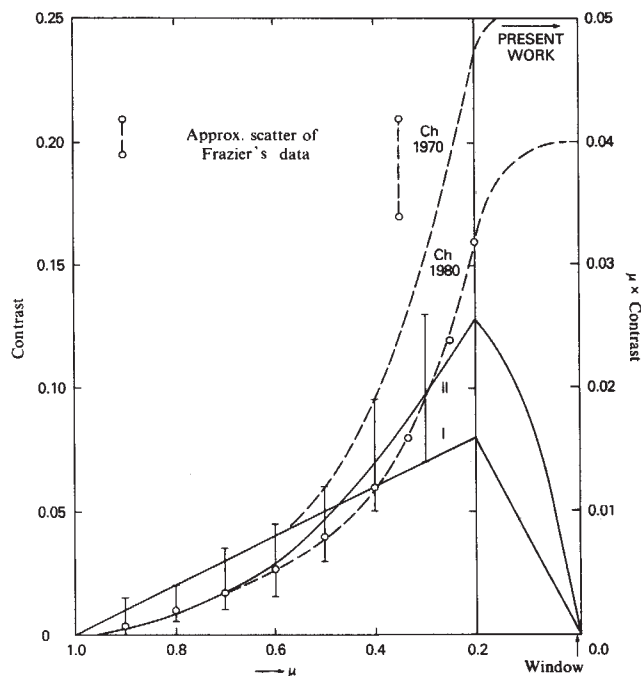


Fig. 1 Centre-to-limb variation of facular contrast for observations and models of Frazier⁸ (○), Chapman^{7,9} (dashed lines) and Sofia *et al.*⁶ (solid lines—left panel). Right panel shows the same models, however, the contrast is multiplied by μ , the cosine of the angular distance from the Sun's centre, to allow the limb values to be seen. The oblateness observations reflect solar conditions at a window (shown) close to $\mu = 0.01$. In the present analysis (arrow at top), a facular contrast within the range of acceptable values allows an acceptable fit to the oblateness measurements (see Fig. 2).

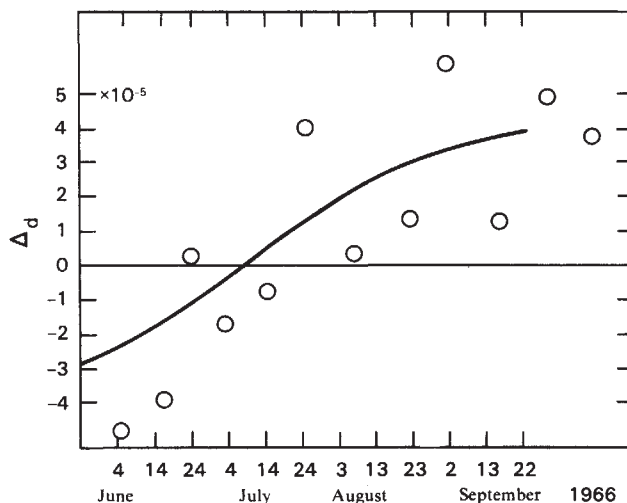


Fig. 2 Solid curve is the Dicke and Goldenberg best fit to the diagonal component of the solar oblateness (Δ_d). ○, The facular contributions to an apparent oblateness calculated from a Chapman $1/\mu$ contrast model.

markedly for these models. As the facular influence on the apparent oblateness involves the contrast near $\mu = 0.01$ (shown by the arrow marked for the oblateness window), the apparent oblateness depends strongly on the form of the contrast function.

If we choose equation (3) and use a contrast coefficient (near 0.05) shown by the 'present work' arrow in Fig. 1, our oblateness

calculation provides an apparent oblateness shown in Fig. 2 (open circles). These results are similar to the oblateness findings (solid curve) of Dicke and Goldenberg.

Dicke⁵ felt that faculae could be ruled out because the values of the oblateness measurements were independent of window size. Equation (1) has a facular contribution which varies with window size (proportional to μ). However, the $1/\mu$ term in the Chapman facular contrast, cancels the μ factor on the left, and the resulting apparent oblateness from facular contributions is independent of window size (see Fig. 1).

The functional form of the facular contrast provides insight into the origin of the emission. The $1/\mu$ form for the facular emission could occur from an optically thin gas sitting above the photosphere. Spruit¹⁰ provides strong evidence that the facular emission comes from the inner hot walls of unresolved thin flux tubes. In accordance with observations, this provides their greater contrast when they are viewed at a large angle. The emission from this geometry does diminish at the limb, as in our equation (1) (see Fig. 1). Thus from Spruit's model, the

lower value for the facular emission seems to be favoured. It does seem possible, however, that the facular emission is the cause of a considerable fraction of the oblateness signal. Thus in the present analysis, a facular contrast within the range of acceptable values allows an acceptable fit to the oblateness measurements.

Note that Dicke (personal communication) is repeating his oblateness experiment, with a view towards examining the higher-numbered even harmonics of the limb shape. The facular contribution and the oblateness contribution to these higher harmonics have a differing behaviour. The faculae, assuming they are fairly localized on the Sun, provide a nearly flat harmonic spectrum diminishing only in accordance with the associated facular patch size. The contribution from a true oblateness source, however, decreases to near zero with harmonics >2 , in accordance with the Fourier transform of the Maclaurin ellipsoid shape. Thus the new observations may allow a non-controvertible separation between these two components.

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Two-dimensional reconstructions from one-dimensional data by maximum entropy

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The maximum entropy method (MEM) is a powerful information-theoretical approach to the inversion of many types of data in science and engineering. It has been used for reconstructing images in such areas as radioastronomy^{1,2}, medical tomography³ and crystallography⁴. Practical data are always corrupted by noise and are usually incomplete; for example, there may be missing projections or missing Fourier components. Also, the instrumental response function may be incompletely known⁵. These factors can lead to severely ill-posed inverse problems. The method is used here in a novel technique for analysing time-series data representing a set of non-interacting damped oscillators. The time series is simultaneously analysed in both frequency and decay to obtain positive two-dimensional reconstructions on the frequency-decay plane. The method is shown to be superior to conventional techniques in that it allows frequency and decay information to be more reliably extracted from the data. Such data occur in areas such as Fourier transform nuclear magnetic resonance (FTNMR), which is used here as an example. In the FTNMR case the decay is the inverse of the effective spin-spin relaxation time.

The FTNMR experiment consists of perturbing the magnetic moments of a chemical compound with a transient magnetic field and observing the profile of oscillating decays as the moments relax to their equilibrium configuration in an underlying uniform field. The data are a time series representing the overall magnetic moment, from which one wishes to recover the frequencies and decays of the individual chemical species in the compound. The early data points in the time series frequently need to be rejected because of distortions due to finite pulse width and thus the data are incomplete. There is also usually an imperfectly known time delay before detection and an imperfectly known misalignment angle between the detector and the net magnetic moment at time $t = 0$. MEM can

recover these parameters as well as find both the frequencies and decays, even though the recovery of decays is a notoriously ill-posed problem.

Our problem is of the novel type

$$F(t) = \int_0^\infty dk e^{-kt} \int_{-\infty}^\infty d\omega e^{-i\omega t} f(k, \omega) \quad (1)$$

in which a two-dimensional function $f(k, \omega)$ representing a set of oscillators of strength $f d\omega dk$ in the frequency-decay cell $d\omega dk$ produces a one-dimensional time series $F(t)$. The data D_t are noisy samples

$$D_t = \text{Re}(F(t)) + r_t \sigma_t \quad (2)$$

where σ_t is a standard deviation and r_t is random with unit variance. From D_t one wishes to reconstruct $f(k, \omega)$.

The conventional technique is based on Fourier transforming D_t . An individual species μ of frequency ω_μ and decay k_μ would give a spectrum whose real positive frequency part is a lorentzian

$$u_\mu(\omega) = f_\mu k_\mu / (k_\mu^2 + (\omega - \omega_\mu)^2) \quad (3)$$

peaked at ω_μ with width k_μ . The corresponding imaginary part is a dispersion mode of form

$$v_\mu(\omega) = f_\mu (\omega - \omega_\mu) / (k_\mu^2 + (\omega - \omega_\mu)^2) \quad (4)$$

This part of the spectrum is rejected. The relevant part of the spectrum is the sum of lorentzians $u(\omega) = \sum_\mu u_\mu(\omega)$ from which it is intended to extract f_μ , ω_μ , k_μ , for each species. However, close rapidly decaying components can overlap strongly and become confused. Apodization techniques^{6,7} may be used to improve resolution. For instance, if the decays are all the same, equal to k_0 say, then multiplying $F(t)$ by $\exp(k_0 t)$ and then Fourier transforming yields, ideally

$$\tilde{u}_\mu(\omega) = f_\mu \delta(\omega - \omega_\mu) \quad (5)$$

But this is unsatisfactory when the decay rates differ and, in any case, a simple exponential filter is liable to give large noise amplification. Noise may be suppressed by modifying the filter. For example, $\exp(k_0 t - Ct^2)$ where $C > 0$ is often used but suppressing noise leads to poorer resolution. In general there is no all purpose apodization filter.

To implement MEM one first constructs the χ^2 -statistic

$$C(f) = \sum_i (\text{Re}(F(t_i)) - D_i)^2 / \sigma_i^2 \quad (6)$$

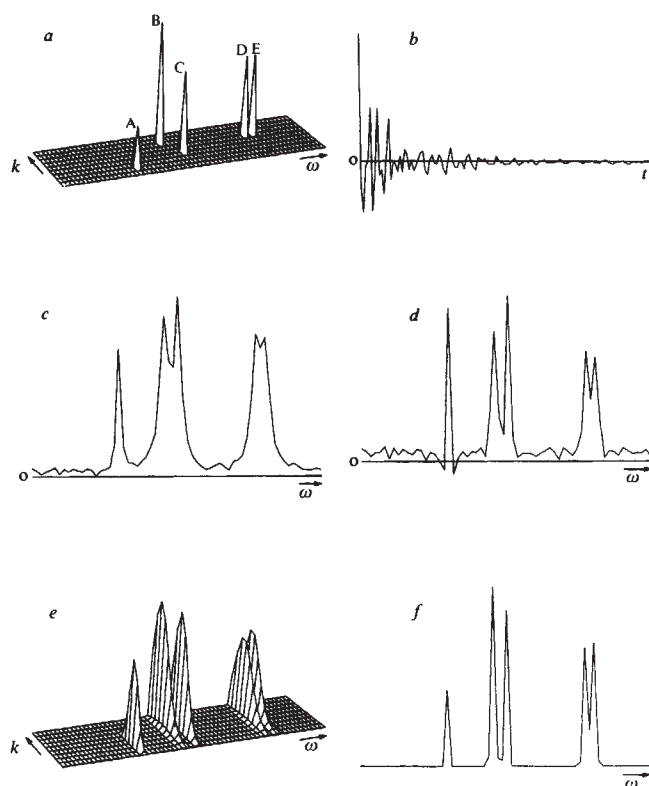


Fig. 1 *a*, Simulation. *b*, Corresponding time-series data. *c*, Conventional reconstruction: Fourier transform of time series. *d*, Fourier transform of apodized time series. *e*, MEM two-dimensional reconstruction. *f*, Projection of *e* to frequency axis.

and defines the set $E = [f: C(f) \leq C_0]$ as the 'feasible' set reconstructions consistent with the data, where C_0 is some chosen tolerance level (say, the value corresponding to 99% confidence). Any f for which $C(f) > C_0$ is rejected: its residuals are too large for it to be consistent with the data. However, one still needs to select just one positive reconstruction from the large set E . To this end it is necessary to introduce an appropriate regularizing function. The regularizing function used here is the entropy of the reconstruction.

The configurational entropy of a reconstruction is⁸

$$S(f) = - \int dk \int d\omega p(k, \omega) \log(p(k, \omega)/m(k, \omega)) \quad (7)$$

where

$$p(k, \omega) = f(k, \omega)m(k, \omega) / \iint dk d\omega f(k, \omega)m(k, \omega) \quad (8)$$

Here, $m(k, \omega)$ is the underlying measure in the (ω, k) plane. The global (unconstrained) maximum of S is attained when $f(k, \omega)$ is uniform. Maximizing S subject to the feasibility constraint yields that unique reconstruction which has least overall structure relative to $m(k, \omega)$. Spurious structures due to noise and instrumental artefacts are automatically suppressed¹.

To determine m , we observe that the natural measure in the frequency-decay-time-phase plane (ω, T) is uniform—there is an uncertainty relation

$$\delta\omega \delta T = \text{constant} \quad (9)$$

between ω and $T = k^{-1}$. This transforms to a measure

$$m(k, \omega) = |\partial(\omega, T)/\partial(\omega, k)| = \text{constant}/k^2 \quad (10)$$

in the (ω, k) plane. MEM can now be used to find the optimal two-dimensional reconstruction $f(k, \omega)$ which fits the data. In previous applications of MEM mentioned above, the natural measure in the reconstruction space is uniform.

The method is being used on real FTNMR data (typically 8,000 points), but here simulated results are reported. In each

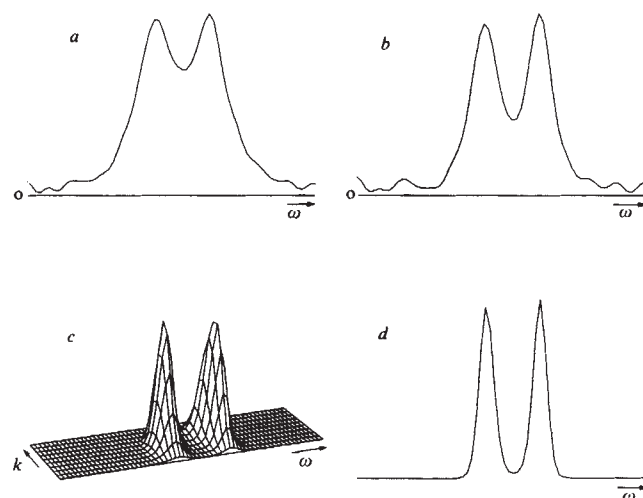


Fig. 2 *a*, Fourier transform of time series. *b*, Fourier transform of apodized time series. *c*, MEM two-dimensional reconstruction. *d*, Projection of *c* to frequency axis.

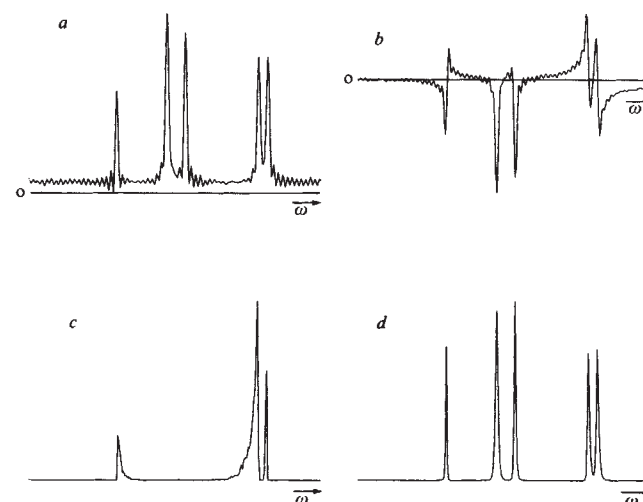


Fig. 3 *a*, Fourier transform of time series with no phase imperfections. *b*, Fourier transform of time series with phase parameters $t_0 = 1.8$, $\psi = 0.4$. *c*, MEM reconstruction of the time series of case *b*: solution of equation (12) at $t_0 = \psi = 0$. *d*, MEM reconstruction at the obtained phase parameters $t_0 = 1.63$ and $\psi = 0.64$.

case the data are noisy with noise of unit standard deviation (relative to a total amplitude $\iint f(k, \omega) dk d\omega = 200$). Figure 1*a* shows an example of five components of different decays as a two-dimensional plot against decay k and frequency ω . Figure 1*b* is the corresponding time-series data sampled at 128 points. Figure 1*c* is the conventional spectrum of Lorentzians. Peak B appears to be of lower amplitude than C because of its faster decay. But, because of the strong overlap, it is not possible to tell which is the broader, let alone deduce the width. Figure 1*d* is the spectrum obtained after applying the apodization filter $\exp(k_0 t - Ct^2)$ to the data. Here, k_0 is the decay of the peaks D and E and $C = k_0/T_0$ where T_0 is the length of the time series. Noise has been amplified and the amplitude ordering is clearly wrong.

The MEM two-dimensional reconstruction is shown in Fig. 1*e*. Peaks are well-resolved in ω and the decay values may be read off the k axis although peaks are broad along this axis. This is because of the inherent difficulty of recovering decays. The width in k gives the substantial uncertainty in the decay values whilst the small width in ω shows that each frequency is well-determined.

Figure 1*f* is the projection of Fig. 1*e* onto the frequency axis. By comparison with Fig. 1*c* and *d*, the peaks are much better

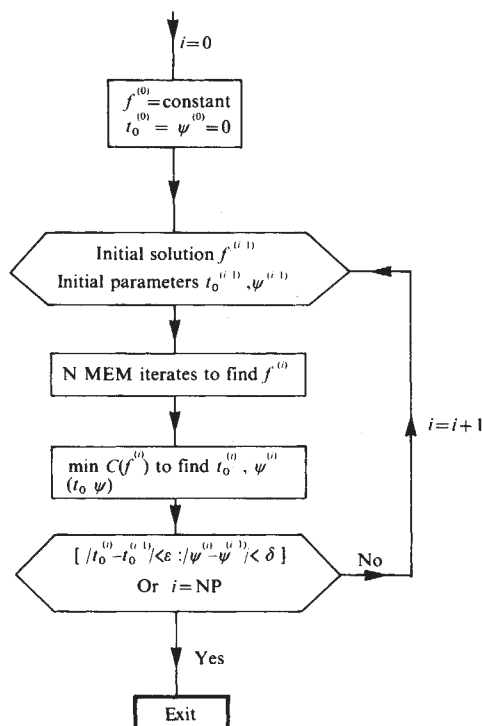


Fig. 4 Phasing flow chart. ϵ and δ are chosen tolerance levels. NP is the maximum number of phasing iterates.

resolved and the ordering of amplitudes has been corrected. The wavy background in Fig. 1c and d due to noise is absent from Fig. 1f. Thus, even when decay information is removed by projecting onto the frequency axis, MEM is still inherently superior to conventional apodization because it allows each component to be fully resolved in ω , regardless of its decay rate. Even for the simple case of peaks of the same decay MEM reconstructions are better. Figure 2 treats a case of two peaks of the same decay together with the result of using a matched exponential apodizing function.

Note that this analysis is possible because of the relative sparseness of the components; it is not realistic to hope to recover more than about one component in the decay axis for a given frequency. After all, one does only have one-dimensional data. The method, however, remains relevant for FTNMR and for problems in engineering which have relatively few modes of oscillation.

As already mentioned, with practical FTNMR data there are imperfectly known phase parameters: the delay time t_0 and the constant angle ψ . The real part of the Fourier transform of the data is now given by

$$\tilde{u}_\mu(\omega) = \cos(\omega_\mu t_0 + \psi)u_\mu(\omega) + \sin(\omega_\mu t_0 + \psi)v_\mu(\omega) \quad (11)$$

There are undesirable dispersion terms in the spectrum. The two-dimensional solution of equation (1) will similarly have frequency derivative terms leading to distorted amplitude, frequency and decay values. To reconstruct correctly, it is necessary to find these parameters and correct the instrumental response function.

The full power of two-dimensional reconstruction is not needed merely to find two parameters. The problem may be simplified by suppressing the integration over k to solve

$$F(t) = \int_{-\infty}^{\infty} \exp[-i\omega(t+t_0) - i\psi]f(\omega) \quad (12)$$

The nonlinear behaviour of the entropy is used to recalibrate these instrumental parameters. One must choose initial phase parameter values, say $t_0 = \psi = 0$. Because of entropy, the reconstruction is forced to remain positive despite the presence of derivative terms, so the data are fitted rather poorly. Having

obtained this initial reconstruction, it becomes clear that the data could be fitted better by adjusting t_0 and ψ . Specifically, one minimized χ^2 over t_0 and ψ . The obtained parameters may then be used in equation (12) to generate a better MEM reconstruction. This process may be repeated until the change in successive parameter values is small. In this way, the MEM reconstruction is iterated to the correct reconstruction which has no derivative terms. This procedure can be readily automated so that one need only specify the initial parameter values.

A time series of 512 data points and no phase imperfections was simulated. Figure 3a is the corresponding spectrum. Non-zero phase parameters were then introduced into the time-series with $t_0 = 1.8$ in units of the sampling interval and $\psi = 0.4$ rad. This time-series yields the spectrum in Fig. 3b. Figure 3c is the MEM reconstruction, for the latter time series, at $t_0 = \psi = 0$. The spectrum is everywhere positive which is the essential feature for automatic phasing. The phasing procedure yielded $t_0 = 1.63$ and $\psi = 0.64$ rad and Fig. 3d is the MEM reconstruction at these parameter values. It is clear that this spectrum is well-phased; it is a spectrum of Lorentzians as desired. By comparison to Fig. 3a, the noise in Fig. 3d has also been suppressed. Figure 4 shows a flow-chart of the automated phasing procedure.

The parameters obtained may then be used to find the two-dimensional solution of the modified equation

$$F(t) = \int_0^\infty dk \exp[-k(t+t_0)] \times \int_{-\infty}^{\infty} d\omega \exp[-i(\omega(t+t_0) + \psi)]f(k, \omega) \quad (13)$$

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Geomagnetic westward drift using the correlation coefficient

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The westward movement in time of features of the pattern of the geomagnetic field on the surface of the Earth¹ is termed the 'westward drift' and is supposed to occur because the Earth's outer, liquid core, where the main geomagnetic field originates, rotates more slowly than the mantle. The drift rate is not constant and it has been suggested² that changes in the rotation rate of the core, as seen in the variation of westward drift, will be associated with changes in the rotation rate of the mantle, that is, in the observed length of day. Le Mouél *et al.*³ have recently presented evidence in support of this theory. Here I attempt to derive an estimate of westward drift from global models of the magnetic field, to determine whether a correlation can be found from the data. The conclusion reached is much less optimistic than that of Le Mouél *et al.*³ as the changes in length of day do not appear to follow closely the values of westward drift obtained.

The observed movements of the geomagnetic field indicate a more complex core motion than a simple westward drift^{4,5}, although the details are far from being well determined; nevertheless a large part of the observed field changes may be attributed to westward drift. For obscure reasons the drift rate of the centred dipole part of the magnetic field is much less than that of the non-dipole field⁶ and, following previous practice⁷, consider here the westward drift of the non-dipole field.

As the westward drift does not account for all the changes in pattern of the non-dipole field, and as estimates of the field pattern are subject to errors, a rotation about the geographical axis will not, in general, produce exact coincidence between the patterns for different epochs. The method used here is to take two geomagnetic field models for different epochs and to calculate the coefficient of correlation between the later field pattern, and the earlier pattern rotated through successive angles of 0.01° westward. The angle for which the coefficient of correlation is maximum is taken as the best estimate of westward drift between the two epochs. The most convenient way of calculating the correlation coefficient is directly from the spherical harmonic coefficients of the models, using the formulae given by Hide and Malin⁸ in connection with the correlation of gravity and magnetic potentials.

The main field models used for the application of this method are those derived during the calculation of a series of secular variation models for a previous paper⁹. Main field fifth-order models are available from 1902 to 1976 and sixth-order models from 1926 to 1976, at 2-yr intervals. These models have the advantages of being evenly and closely spaced in time, and also that adjacent models incorporate data from the same set of geomagnetic observatories, using nearly all available data since 1901. The length of day variation is a global phenomenon whereas Le Mouél *et al.*³ used magnetic data from one station only, Paris. While some nearby stations follow closely the variations in Paris, the wider data set used here is a better representation of the global field changes.

The angles of rotation giving maximum correlation between pairs of models over the 2-yr intervals were calculated and halved to give estimates of the annual drift rate. The choice of order at which the models were truncated did not greatly affect the calculated drift rates at the surface of the Earth, so full sets of coefficients were used. Figures 1a, b shows the calculated westward drift at the surface of the Earth, from the fifth- and the sixth-order models. Figure 1c shows the calculated drift near the surface of the Earth's core (at radius $R = 3,500$ km from the centre of the Earth), from the fifth-order models up to 1927 and from the sixth-order models for 1927–75. The maximum correlations differ from unity by $<10^{-4}$ in every case, so that the maxima are rather flat, though well defined.

Figure 1a and c both show a minimum followed by a maximum in the first 15 yr of the century and all three graphs show a general decreasing trend in the rate of drift since about 1915. There is some indication that the 1970 'jerk', when there was a sudden change in the global secular acceleration of the magnetic field, is reflected in a change of slope in the westward drift but more recent data are needed to confirm that the apparently increasing trend since about 1971 is persisting. The values found here for the westward drift are smaller than other estimates based on the eccentric dipole motion, some of which are listed in ref. 9.

The connection with the length of day depends on the conservation of angular momentum for a rotating Earth. A discussion of the mechanism may be found in, for example, ref. 6. In general, a shortening of the length of day would correspond to an increase in westward drift and vice versa. Figure 1d shows the excess length of day in milliseconds as given for each year by Morrison¹⁰, the values being smoothed in the same way as the data for the geomagnetic field models, that is weighted means of three annual values giving half-weight to the two extreme values, differenced to give the change in the length of day. There are several factors which can influence the length of day but none of these investigated have been capable of

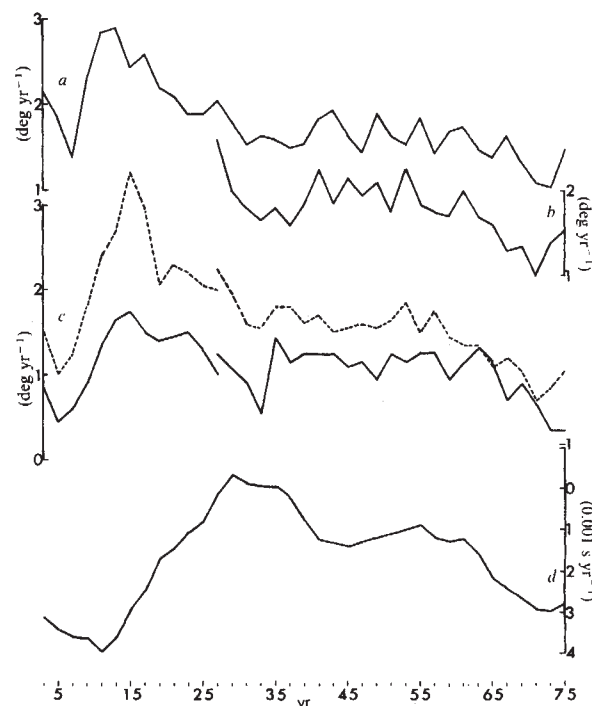


Fig. 1 a, Westward drift at the Earth's surface calculated from fifth-order models; b, westward drift at the Earth's surface calculated from sixth-order models; c, westward drift near the core surface from fifth-order models up to 1927, sixth-order models from 1927, without truncation (solid line) and truncating at fourth order (dotted line); d, change in length of day measurements.

producing changes of observed magnitude (see, for example, ref. 11), leaving fluctuations in core rotation as the only apparent possible cause. However, the results given here do not supply any striking positive evidence for this link. If the turning-points in the length of day curve correspond to those in the westward drift curve, not only is there a time-lag but it is non-uniform. Note that the association with westward drift depends on the field pattern being fixed in the Earth's outer core and rotating at the same rate. Hide¹² has shown that magnetohydrodynamic waves within the core can account for the westward drift if certain reasonable assumptions are made, without involving westward motions of the core material. Therefore, we conclude that, while there may be a connection between core motions and changes in length of day, it is not likely that this will be clearly shown in the westward drift, and indeed is not shown in drift variations derived from the complete set of available geomagnetic measurements since 1901.

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Inhomogeneity parameter of a homogeneous Earth

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Adiabatic and chemically homogeneous solid regions of the Earth are expected to appear otherwise because of the bulk attenuation to seismic waves that occurs in polyphase aggregates such as the Earth's mantle. Because the seismologically measured bulk modulus is only partially relaxed compared with the hydrostatic pressure-density relation (or relaxed modulus) of the Earth's mantle, an apparently nonadiabatic contribution¹ to the geotherm is expected to be inferred from seismological observations on regions of the mantle that are well mixed by vigorous convection. Using a recent analysis of bulk attenuation in polyphase assemblages², we estimate the total nonadiabatic contribution to the geotherm through the lower mantle to be approximately -300 to -800 K. The magnitude of this effect is significant in that it is of the same order as the temperature change within a thermal boundary layer, which represents the largest perturbation of the geotherm resulting from convection within the Earth. Moreover, if the lower mantle is truly homogeneous and adiabatic, it should appear to be subadiabatic according to the seismological data.

Based on seismological observations³⁻⁵, the Earth's lower mantle appears to be homogeneous and adiabatic. This is presumably a reflection of the importance of convection for transferring heat out of the deep interior of the planet⁶⁻⁸. Brown and Shankland^{9,10} have recently studied the homogeneity of the lower mantle in detail, and suggested that small-scale regions in which seismological models do not satisfy the Adams-Williamson equation could be interpreted in terms of non-adiabatic contributions to the geotherm (specifically, thermal boundary layers). Local deviations of the temperature field from adiabaticity are intimately linked with the nature of the flow pattern within the mantle. Therefore, the conclusion that nonadiabatic regions can be identified by seismological observations is important for placing constraints on the present thermal and dynamic state of the Earth's interior. High-pressure phase equilibrium studies demonstrate, however, that the phases that are expected to coexist in the lower mantle have widely differing elastic moduli, and should therefore lead to significant bulk attenuation². This mechanism results from the internal shear stresses that are generated in the aggregate due to the local mismatch of the elastic moduli of neighboring grains. The result is that the Adams-Williamson equation is no longer satisfied for the composite, even in adiabatic conditions, and consequently Bullen's inhomogeneity parameter¹¹ deviates from unity [or "one" or "1" or "a value of 1"].

The inhomogeneity parameter is defined as:

$$\eta = \frac{\phi}{\rho g} \frac{d\rho}{dr} \quad (1)$$

where ρ is the density, r is the radial distance from the centre of the Earth, g is the acceleration of gravity, and ϕ ($=K_s/\rho$) is the seismic parameter (K_s is the adiabatic bulk modulus). Seismological observations constrain η directly, and the average value in the lower mantle is resolved to ~2% (ref. 12). The right-hand side of equation (1) is equivalent to the ratio K_s/K_E , where K_E is the effective bulk modulus (local pressure-density derivative) within the Earth. The appropriate modulus to use for K_E is the Reuss bound^{13,14} because the mantle is under hydrostatic pressure: over tectonic time scales shear strains produced by the impingement of neighbouring grains are relaxed¹. The adiabatic bulk modulus which determines the

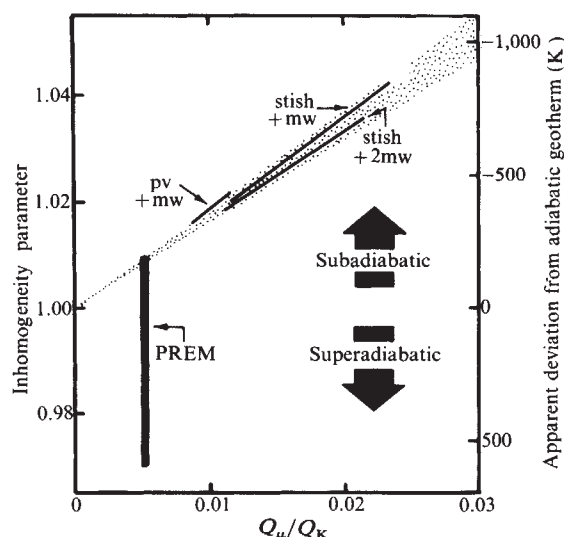


Fig. 1 The theoretical correlation of the inhomogeneity parameter and bulk attenuation within the solid Earth. The attenuation is due to an aggregate mechanism that is caused by the differing compressibilities of coexisting minerals. The shaded area represents the range of values expected for the lower mantle based on the results derived for three, specific assemblages involving the high-pressure phases perovskite (pv), magnesiowüstite (mw) and stishovite (stish): straight lines in the shaded area are calculated for pressures over the interval 0 (high Q_μ/Q_K end) to 130×10^9 Pa (low Q_μ/Q_K end). For each phase, the experimentally determined values of the elastic moduli are used (the effects of intrinsic bulk attenuation and of anisotropy are ignored)². The bar 'PREM' is from a recent whole Earth model that includes bulk attenuation⁴. The right-hand axis is the apparent nonadiabatic contribution to the geotherm evaluated over the lower mantle, as discussed in the text.

velocity of seismic waves, K_s , differs from K_E , either when the polycrystalline medium contains elastically anisotropic grains, or when the medium consists of more than one phase. Consequently, the inhomogeneity parameter is expected to deviate from unity in most regions of the solid Earth, even where these are adiabatic and homogeneous (note that by adiabatic we mean that there is negligible heat flow over the length-scale of the probe: that is, over seismic wavelengths; thermoelastic and other mechanisms of bulk attenuation are ignored in this analysis).

The straight lines in the shaded area of Fig. 1 represent the theoretical correlation of bulk attenuation with the inhomogeneity parameter for several petrological assemblages of candidate lower mantle phases²: perovskite (pv), magnesiowüstite (mw) and stishovite (stish). The bulk attenuation, as well as the inhomogeneity parameter, depends on the maximum difference in the elastic moduli of the constituent phases, which is typically on the order of tens of per cent. In each case, the complex moduli of the composite have been calculated using the self-consistent scheme in accord with viscoelastic theory¹⁵⁻¹⁷. The right-hand scale of Fig. 1 indicates the apparent deviation from an adiabatic geotherm in the lower mantle, corresponding to η differing from unity "a value of one" [viz. note above]. This has been calculated from:

$$dT_s - dT_E = \frac{(\eta - 1)g\rho}{\alpha K_s} \Delta r \quad (2)$$

where α is the volume coefficient of thermal expansion, and $\Delta r = 2,000$ km is the thickness of the lower mantle. The values of $\alpha \cong 10^{-5} \text{ K}^{-1}$, $g \cong 10 \text{ m s}^{-2}$, $\rho = 5.0 \times 10^6 \text{ g m}^{-3}$, and $K_s \cong 5 \times 10^{11} \text{ Pa}$ were used⁸. The effect of bulk attenuation is always to produce an apparently lower geothermal gradient than actually exists (Fig. 1); under the right circumstances an apparently subadiabatic geotherm could, in reality, be superadiabatic. Conversely, if there is an *a priori* reason for believing

that any regions within the solid Earth are in fact homogeneous and adiabatic, then these should be taken to be slightly sub-adiabatic (for example, $\eta \approx 1.02$ – 1.03 ; Fig. 1) in the starting models that are used for the inversion of seismological data.

In conclusion, it is not useful to use the inhomogeneity parameter to distinguish departures from adiabaticity unless the effects of bulk attenuation can be explicitly determined. This is because departures from $\eta = 1$ due to bulk attenuation are significant compared with the deviations from adiabaticity that are expected in the convecting Earth. In this sense, a detailed knowledge of the differences in elastic properties among coexisting minerals is required to use seismological data to place constraints on the thermal state of the mantle.

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Location of a magma reservoir beneath Hekla Volcano, Iceland

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Hekla, situated on the western boundary of the eastern volcanic zone in South Iceland, is one of the most active volcanoes in Iceland having erupted 16 times since 1104. Repeated electronic distance measurements since its eruption in 1980–81 show significant changes in crustal movement in the aftermath of the eruption. Such movement on and around volcanoes can be used to monitor movement of magma within and below volcanoes^{1–3}. We show here that these measurements can be interpreted in terms of inflating magma reservoirs with a centre at ~8 km depth below the volcano. Over a period of 10 months, the volume of the inflation amounted to $55 \times 10^6 \text{ m}^3$.

Hekla forms a ridge rising to ~1,500 m elevation or up to 1,000 m above the surroundings. Its eruptions produce intermediate⁴ rock type. Although basaltic eruptions have taken place in the vicinity of the volcano (the last one being in 1913) these eruptions are not included as Hekla eruptions.

The volcanic history since 1104, the first eruption recorded, has been characterized by relatively short eruptive periods interrupted by long periods with no visible activity that last up to 101 yr. Eruptions start suddenly with eruption periods lasting up to 2 yr, during which activity may virtually stop for months. The vigour of the initial eruption and the final volume of the tephra and lava, as well as the acidity of the initial product, seems to be related to the length of the preceding quiet intervals. The most recent eruptions of Hekla took place in 1947–48⁵, 1970⁶ and 1980–81.

Table 1 Distances and elevation differences between stations measured on 17 May 1981

Stations	Elevation difference (m)	Distance (m)	Distance change (m)	Computed change (m)
HB SF	–180.10	5,396.028	0.014	0.014
HB SB	–362.34	4,601.243	0.039	0.039
HB VF	53.45	6,579.158		
VF HH	7.08	4,201.950		
VF RK	24.74	7,313.694		
VF SF	–233.54	10,133.185		
VF MF	–15.26	5,921.632		
HH MF	–22.34	4,592.722		
RK MF	–40.00	3,599.315		
RK HA	–70.37	5,542.385		
RK NB	–421.60	5,709.495		
RK SB	–440.53	8,183.974	0.081	0.080
HA MF	30.37	6,881.839		
HA NB	–351.23	7,312.044		
SB NB	18.92	5,843.705	0.037	0.038
SB SF	182.24	8,045.351	0.059	0.060

The elevation differences have been adjusted by a least-squares method. The distances listed are distances between benchmarks measured. In no case did a least-squares adjustment of the distances result in a change of more than 1 mm. Also shown is the lengthening for the lines that were measured on 18 March 1982. The last column shows the distance changes resulting from the Mogi solution given in Table 2.

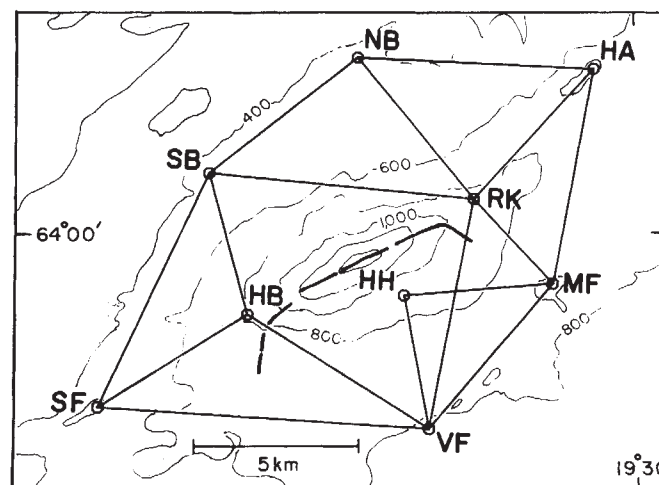


Fig. 1 The location of the benchmarks and distances measured 17 May 1981. The heavy line delineates the fissure that erupted in August 1980. Elevation contours are at 200-m intervals.

On 17 August 1980 an intense tephra eruption began. It lasted for a few hours and was closely followed by lava emission that carried on with decreasing vigour until 20 August. The lava was erupted from an 8-km long fissure that split the mountain. The volume of lava was estimated at $100 \times 10^6 \text{ m}^3$ and the tephra at $20 \times 10^6 \text{ m}^3$ calculated as solid rock. The volcano then remained quiet, except for continuous steam emission. On 9 April 1981 activity resumed, this time with mainly lava emission from the top crater producing about $20 \times 10^6 \text{ m}^3$, and lasted until 16 April. The chemical composition of the lavas from both the eruptions and the early tephra is very similar. Comparing the pattern of this eruption with earlier ones suggests that it can be considered a single eruption episode. It is, in most respects, a typical Hekla eruption although the repose interval which lasted from 1970 is the shortest in the observed history of the volcano. However, Wickman's statistical Markov model for the activity of Hekla features two repose intervals of <20 years during several hundred years of activity⁷.

On 17 May 1981, the Nordic Volcanological Institute carried out distance measurements on a net with nine benchmarks using

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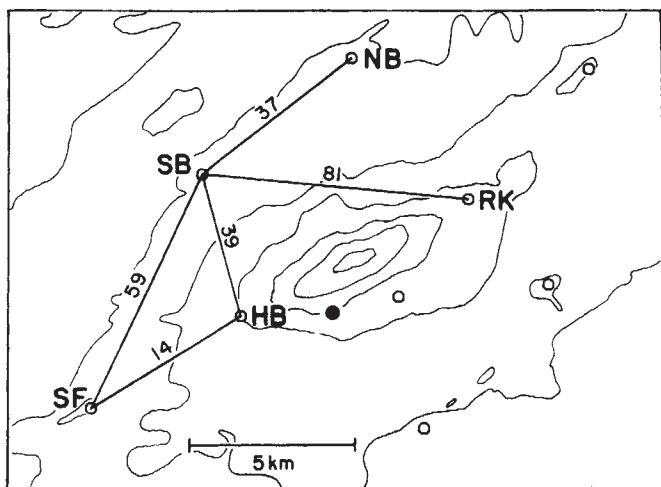


Fig. 2 The distances measured 18 March 1982. ○, The benchmarks used in 1981. ●, The location of the centre of inflation given in Table 2. The increase of the distances is shown in mm.

an AGA Geodimeter model 6BL. The 16 distances measured are listed in Table 1 and the net of benchmarks and distances are shown in Fig. 1. The lines measured were used to compute coordinates in a local frame for the benchmarks used. As only 15 lines are needed to constrain the relative coordinates of the stations, the fit of the measurements provides some measure of their precision. This indicated a standard deviation of 2 mm for the individual distance measurements. The manufacturers specifications give a standard error of 5–10 mm for measurements of distances in the range 4–10 km.

Five of the distances were remeasured on 18 March 1982: all of the lines showed lengthening, ranging from 14 to 80 mm (see Table 1 and Fig. 2). The lengthening of the distances is significantly greater than the error limits given by the manufacturer. An attempt to reoccupy the whole network in May 1982 was frustrated by instrument malfunction.

Crustal deformation observed on or near Krafla^{2,8} and Kilauea³ has been shown to be related to both rifting associated with injection of magma into dykes, and the bulging or subsidence of a circular region related to magma movement into or out of localized magma reservoirs.

Distance measurements, carried out by Decker *et al.*⁹ before and after the 1970 Hekla eruption, on a profile 20 km to the north-east of Hekla, showed no significant rifting. Following the 1980–81 eruption no movement of faults on the continuation of the eruptive fissure could be observed. Thus significant rifting was mainly confined to the volcanic edifice. A precision levelling station 4 km north of the Hekla summit showed large movement at the time of the 1970 eruption (E. Tryggvason, personal communication).

A circular uplift connected with the expansion of a localized magma reservoir is usually interpreted in terms of a model introduced by Mogi¹. This gives a solution for the surface deformation of an elastic half-space which includes a nucleus of expansion (or contraction). The solution describes deformation at the surface which is radial away from the source and the magnitude of the deformation vector is inversely proportional to the distance from the source.

An attempt to fit the distance changes measured between May 1981 and March 1982 to a simple Mogi type model, yielded surprisingly consistent results. A model based on a single nucleus of expansion has four parameters, three for the location and one for the volume of the uplift. A solution determined using linearized iterative least squares was found that satisfied all of the measured distance changes with a residual of 2 mm or less. The parameters of the solution are shown in Table 2. When any one of the measurements was omitted, a solution was obtained that fell within the bounds shown.

Table 2 Features of the Mogi model that gave best fit to the distance changes

Depth to the inflation centre	7.7 ± 0.5 km
East coordinate	± 0.2 km
North coordinate	± 0.1 km
Maximum uplift	147 ± 3 mm
Volume change	55 ± 8 × 10 ⁶ m ³
Maximum tilt	16 × 10 ⁻⁶ rad

Table 1 shows the computed distance changes that result from this solution. The error limits were estimated by observing the effect of omitting any of the five lines in the calculation of the parameters for the Mogi model.

Although the data thus show excellent agreement with the Mogi model, the actual shape of the source is quite unconstrained. A sill or dyke a few kilometres across could result in virtually the same deformation at the surface.

The most straightforward interpretation of this result is that during the 10 month study period, ~55 × 10⁶ m³ of magma flowed into magma reservoirs with a centre of inflation at a depth between 7 and 8.5 km, ~2 km south of the summit of Hekla.

This volume is more than double that erupted in the April eruption 1981 but it is less than half of what was erupted in August 1980. As we have no data for the behaviour between eruptions, it is not known whether the April eruption represented new magma recently fed into the reservoirs, or leftovers from the previous eruption, nor whether different reservoirs were operating in the two eruptions. The similarities in chemical composition and the fact that the volcano has already recovered more than the volume erupted in the April eruption supports the contention that these two eruptions can be considered as a single eruptive episode.

The average supply rate for the 10 month period between measurements is ~2 m³ s⁻¹. At Krafla in North Iceland where similar inflation rates are observed⁸, these are found to be highest immediately following deflations caused by dyke injection or eruptions, but the rate slows down drastically as previous ground levels are approached. If Hekla behaves in a similar way then a short-term average inflation rate may not be representative of the long term behaviour of the volcano. The average discharge rate for Hekla since 1845 is ~0.3 m³ s⁻¹. Discharge rates for many recently active volcanoes around the world are estimated at 0.3–0.7 m³ s⁻¹ on a similar time scale¹⁰.

The depth to the centre is ~7.7 km relative to the average height of the benchmarks; this need not coincide exactly with the centre of the actual magma reservoirs. This depth is about twice that of the centres at the basaltic volcanoes Krafla and Kilauea, but less than the 10 km depth obtained by Mogi for the andesitic volcano Sakurazima in Japan¹¹. The active volcanoes of Mt St Helens¹¹ and Mt Etna¹² have shown no centres in this depth range. In spite of considerable monitoring the only deformation observed has been focused at very shallow depths.

One of the features that characterizes most central volcanoes in Iceland is that they are associated with high-temperature geothermal activity which is thought to be caused by intrusive magmatic activity. The absence of this type of geothermal activity in Hekla indicates that intrusive activity in Hekla is out of reach of the circulating ground water.

The intermediate and acid magmas that have been erupted from Hekla have been interpreted as products of fractional crystallization of a basaltic parent¹³ or by partial melting of a basaltic lower crust¹⁴. The electronic distance measurements in Hekla have demonstrated that observable processes continue inside the volcano, even when there is no visible eruptive activity.

The Icelandic Coast Guard provided helicopter support and most of the staff members at NVI participated in the initial measurements. The measurements in March 1981 were carried out using snowmobiles with the help of Halldor Olafsson. A review by Pall Einarsson improved the manuscript.

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Unusual isotopic variations in Nyiragongo nephelinites

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Nd and Sr isotopic ratios in Nyiragongo nephelinites are fairly constant and close to 'bulk Earth'^{1,2} estimates. By contrast, ²⁰⁶Pb/²⁰⁴Pb variations are the largest ever observed for young volcanic rocks. If the ~500 Myr Pb–Pb age dates—as we believe—a mantle source event, then the inferred parent-daughter element fractionation patterns suggest this to be a metasomatic event. The data indicate that apparently primitive Nd and Sr isotopic compositions can be deceptive: the mantle under Nyiragongo does not seem to be primitive and undifferentiated but experienced, at different times, large-ionic-lithophile element depletion and enrichment. We suggest here that mantle metasomatism, by causing erratic and sometimes severe fractionation of U from Pb, could be responsible for the more complex evolution of the U–Pb system relative to the Rb–Sr and Sm–Nd systems observed in oceanic basalts^{3,4}.

We analysed for the Pb, Sr and Nd isotopic compositions and U and Pb concentrations melilite-bearing nephelinites from the active Nyiragongo volcano (East Africa) and two, probably closely related, small volcanoes (Goma, Bushwaga) which are situated on the south flank of Nyiragongo. These nephelinites are distinctly different from most Virunga rocks, which are leucite-bearing lamproites (high-K and high-Mg volcanic rocks⁵); we report elsewhere⁶ isotopic data for these latter volcanics. The results are given in Tables 1 and 2. There is no

systematic difference between Nyiragongo, Goma and Bushwaga lavas and all will here be referred to as belonging to Nyiragongo. The ¹⁴³Nd/¹⁴⁴Nd ratio for four samples is 0.51271±2 and ⁸⁷Sr/⁸⁶Sr varies only from 0.70457 to 0.70479; they plot within the 'mantle array' in the Sr–Nd isotope diagram, close to 'bulk Earth' estimates^{1,2}. Sr concentrations are high (2,400–3,600 p.p.m.) and Rb/Sr averages 0.05 (ref. 7). In contrast to the fairly constant and seemingly primitive Nd and Sr isotopic ratios, Pb isotopic variations are the most extreme yet reported for young volcanic rocks. Sample C9872 has a ²⁰⁶Pb/²⁰⁴Pb ratio of 60.2–62.2. Inhomogeneities on a hand-specimen scale (C3022) or even between 100-mg fractions of powdered samples (C9958, C9872) are observed. This suggests that the radiogenic Pb and by inference U and Th are highly concentrated in an accessory xenolithic phase which has not completely equilibrated with the melt. In principle, such a phase may have been picked up by the magma from the upper mantle and it may then be regarded as a constituent of the Nyiragongo source; alternatively, it may have originated in the crust and contaminated the magmas with radiogenic Pb, U, and possibly other elements. Identification of the phase may allow this question to be decided and work on this is in progress. The detailed mineralogical description of Nyiragongo lavas by Sahama⁸ suggests that perovskite may be the only suitable mineral. Because perovskite is a common accessory mineral in basic alkaline rocks, it would point to the U and Th enrichment being a feature of the mantle source.

The Pb–Pb age of the U and Th enrichment is dated to 484±28 (2σ) Myr and the Th/U ratio is calculated to 5.0 if it is assumed that the U and Th accepting phase, at the time of its formation or last equilibration, incorporated Pb with an isotopic composition then identical to the Pb of the Nyiragongo upper mantle source. But even if such an assumption is not made and the U and Th rich mineral had not had the same initial Pb isotopic composition as the upper mantle, the age of this U and Th enrichment is still ~500 Myr and Th/U about 5, because the Pb of sample C9872 is so radiogenic that variations in the initial Pb isotopic composition, within limits commonly observed for mantle or crustal rocks, will not much affect the Pb–Pb age.

Sample C9872 has a measured U/Pb ratio consistent with the Pb–Pb age and a reasonable estimate of the initial ²⁰⁶Pb/²⁰⁴Pb ratio derived from the least radiogenic Nyiragongo samples; it seems that this sample (unlike C9958 and C3022) has preserved source U/Pb ratios. Lack of U/Pb fractionation during partial melting is only understandable if, as we suggested above, U and Pb are concentrated in an accessory xenolithic phase.

Several observations argue against crustal contamination being responsible for the extreme Pb isotopic variations: (1) The Pb of sample C9872 is more radiogenic than any Pb found in common crustal rocks; less common rocks such as ~500-Myr old uranium and thorium mineralizations would have to be considered. (2) All samples fall on the Pb–Pb isochrons (Fig. 1), therefore all except the least radiogenic samples would have to be more or less contaminated. Neither are the two very

Table 1 Sr and Nd isotopic compositions and Rb, Sr concentrations for nephelinites from Nyiragongo and related craters, Virunga volcanic field, East Africa

Sample	Rock-type, locality	Rb (p.p.m.)	Sr (p.p.m.)	⁸⁷ Sr/ ⁸⁶ Sr*	¹⁴³ Nd/ ¹⁴⁴ Nd†
C3021	mel-nephelinite, Bushwaga	136	2,866	0.70463±5	0.51270±5
C3022	mel-nephelinite, Nyiragongo	138	3,643	0.70479±5	0.51272±2
C9872	lc-nephelinite, Goma	110	2,421	0.70457±5	0.51271±2
C9877	glassy tuff, Goma			0.70459±4	
C9958	ne-leucitite, Nyiragongo	149	2,779	0.70475±5	
1972	mel-nephelinite, Nyiragongo 1972 crater-lake			0.70465±4	0.51271±3

Rb and Sr concentrations are from ref. 7.

* Normalized to ⁸⁶Sr/⁸⁸Sr = 0.1194 and relative to an E and A standard value of 0.70800.

† Normalized to ¹⁴⁶Nd/¹⁴⁴Nd = 0.7219 and relative to BCR-1 ¹⁴³Nd/¹⁴⁴Nd = 0.51265.

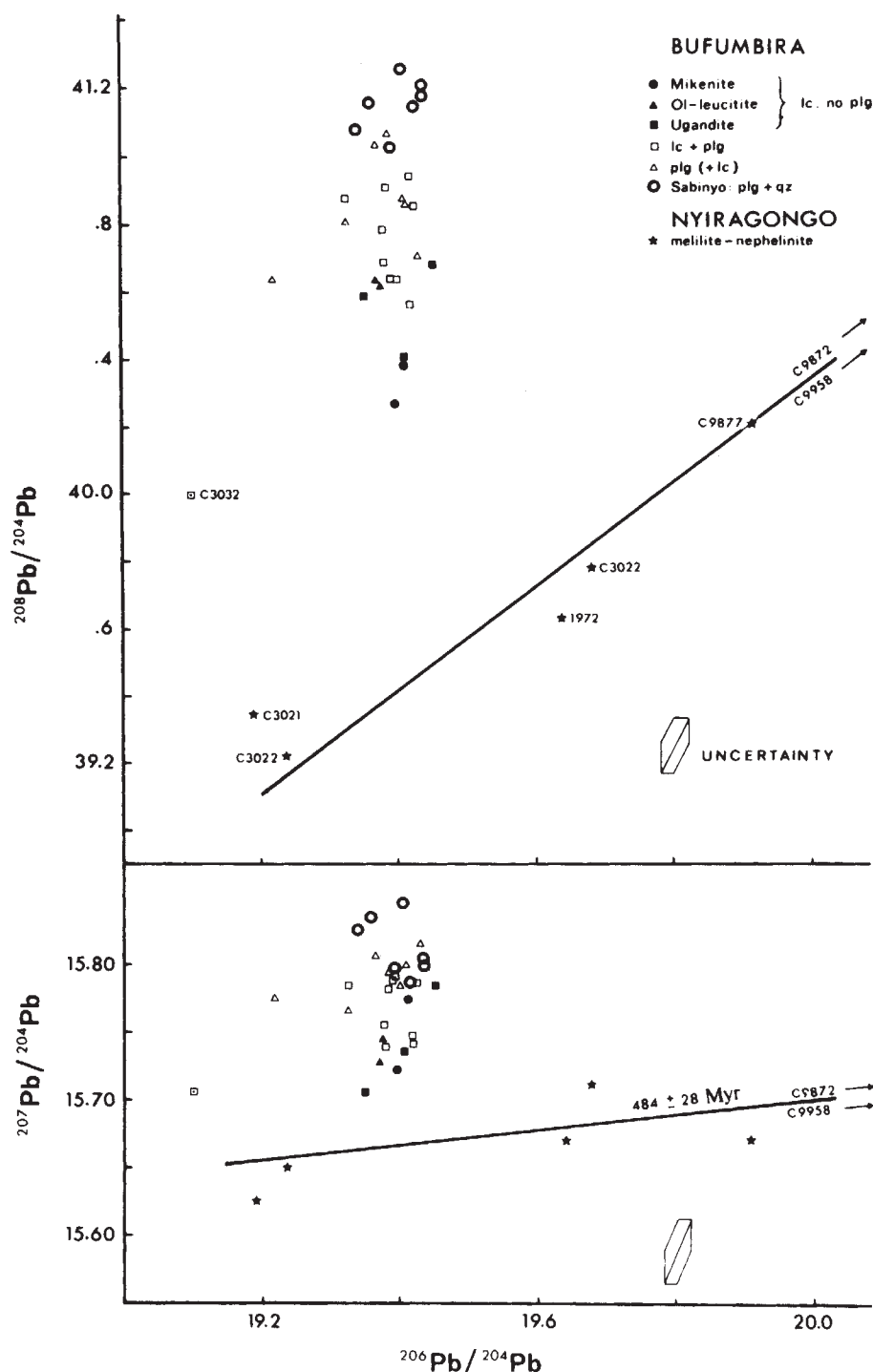


Fig. 1 Pb isotopic compositions of alkaline rocks from the prehistoric-historic Virunga volcanic field, East Africa. Isotopic variations for the Nyiragongo melilite-nephelinites are the most extreme yet recorded for young volcanic rocks; two samples plot on the isochrons at $^{206}\text{Pb}/^{204}\text{Pb} = 23.3\text{--}23.4$ (C9958) and $^{206}\text{Pb}/^{204}\text{Pb} = 60.2\text{--}62.2$ (C9872). The Pb-Pb age of 484 ± 28 Myr (2σ) is interpreted to date a metasomatic event, which variably enriched the Nyiragongo upper mantle source in U and Th relative to Pb and uniformly raised the Th/U ratio to 5.0. Sample C3032 is from Nyamuragira, all others from Bufumbira, the eastern region of Virunga; these data are discussed in ref. 6.

radiogenic samples confined to a small and localized anomaly: one sample (C9872) is from the Goma crater and the other (C9958) from Nyiragongo. Contamination—if it had occurred—would have had to be a widespread and frequent event. (3) If contamination were common for Nyiragongo magmas, it would be the more surprising that there is no evidence from Pb isotopic ratios for crustal contamination of any of the eastern Virunga samples (Fig. 1 and ref. 6). (4) Pegmatitic mineralizations are expected to be high in Rb and consequently to be extremely radiogenic in Sr now; but neither are the Rb abundances anomalous for C9872 and C9958, nor are the $^{87}\text{Sr}/^{86}\text{Sr}$ ratios above the very narrow range established by the other nephelinites (Table 1). In fact, C9872 is the least radiogenic in Sr. However, contamination may conceivably be masked by the very high Sr content of the magmas.

Although the data by no means allow contamination to be excluded, we are inclined to think the extreme radiogenic Pb and the large variations to be a feature inherited by the magmas from their mantle source dating to a source enrichment event.

Such a source event raised Rb/Sr from ~ 0.03 to ~ 0.05 and Th/U from 3.8 (estimated chondritic mantle value⁹) to 5.0. Variations in source Rb/Sr are constrained by the isotopic composition to $<30\%$ and source Th/U variations are likely to be even smaller. By contrast, U/Pb increased—owing to U gain rather than Pb loss (Table 2)—up to a factor of 60. It is doubtful whether such fractionation patterns could be achieved by crystal-melt partitioning in a closed system, as extremely variable amounts of the U and Th accepting phase have been newly formed. We regard this pattern as strong evidence for mantle metasomatism by a fluid which migrated through the

Table 2 Pb isotopic compositions and U, Pb concentrations of nephelinites from Nyiragongo and related craters, Virunga volcanic field, East Africa, and for USGS standard rock BCR-1

Sample	$^{208}\text{Pb}/^{206}\text{Pb}$	$^{207}\text{Pb}/^{206}\text{Pb}$	$^{206}\text{Pb}/^{204}\text{Pb}$	$^{207}\text{Pb}/^{204}\text{Pb}$	$^{208}\text{Pb}/^{204}\text{Pb}$	Pb (p.p.m.)	U (p.p.m.)	$^{238}\text{U}/^{204}\text{Pb}$
C3021	2.0501 $\pm$ 2	0.81416 $\pm$ 5	19.192 $\pm$ 12	15.625	39.346			
C3022	2.0382 $\pm$ 5	0.81326 $\pm$ 16	19.218 $\pm$ 22	15.629	39.171	11.3	13.99	80.0
	2.0392 $\pm$ 2	0.81344 $\pm$ 4	19.254 $\pm$ 8	15.662	39.263			
C3022*	2.0213 $\pm$ 5	0.79830 $\pm$ 15	19.682 $\pm$ 5	15.712	39.783			
C9872†	1.6983 $\pm$ 7	0.29783 $\pm$ 9	60.23 $\pm$ 42	17.94	102.30	14.0	50.3	573
†	1.6959 $\pm$ 3	0.29264 $\pm$ 5	61.75 $\pm$ 18	18.07	104.73			
†	1.6938 $\pm$ 1	0.29077 $\pm$ 3	62.24 $\pm$ 12	18.10	105.42			
	1.6951 $\pm$ 1	0.29085 $\pm$ 2	62.21 $\pm$ 6	18.10	105.46			
C9877	2.0196 $\pm$ 2	0.78689 $\pm$ 6	19.913 $\pm$ 18	15.669	40.215			
C9958†	1.9574 $\pm$ 3	0.68310 $\pm$ 6	23.267 $\pm$ 34	15.894	45.543	7.74	15.39	148
†	1.9554 $\pm$ 3	0.67939 $\pm$ 8	23.421 $\pm$ 26	15.912	45.796			
	1.9576 $\pm$ 4	0.67972 $\pm$ 6	23.436 $\pm$ 10	15.930	45.877			
1972 lava	2.0200 $\pm$ 8	0.79823 $\pm$ 18	19.655 $\pm$ 9	15.689	39.703			
	2.0167 $\pm$ 5	0.79752 $\pm$ 14	19.621 $\pm$ 12	15.648	39.569			
BCR-1	2.0570 $\pm$ 7	0.83066 $\pm$ 20	18.818 $\pm$ 24	15.631	38.710	13.21	1.64	7.96
	2.0556 $\pm$ 3	0.83063 $\pm$ 12	18.797 $\pm$ 8	15.613	38.639			

Procedures for Pb separation, analysis and fractionation correction are identical to those described in ref. 10. Procedure blanks were 1.5–3 ng during the course of the analytical work and are insignificant. Pb isotope ratios for USGS standard rock BCR-1 are within 0.1% of values reported by Tatsumoto *et al.*¹¹ and Arden and Gale¹². Pb and U concentrations were measured by isotopic dilution; values for BCR-1 are slightly lower (by 2% and 5%, respectively) than those reported in ref. 11. Errors are 2σ of the mean and indicate within-run precision.

* Sample-split provided by Keith Bell.

† Separate sample dissolutions.

host rock and deposited or exchanged mobile elements according to their solubility.

The parent–daughter element fractionation patterns inferred here for metasomatism of the Nyiragongo source are the first known example. Should they be the rule rather than the exception, then the Pb–Pb method will be the best way of dating metasomatic events in the mantle. Equally important, meta-

somatism could then possibly provide an answer to the more complex evolution of the U–Pb system relative to the Sm–Nd and Rb–Sr systems which is suggested by the isotopic compositions of oceanic basalts^{3,4}.

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Estimation of evaporation from non-vegetated surfaces using natural deuterium

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Loss of water by evaporation from bare surfaces is difficult to measure, because, like transpiration, it is subject to the vagaries of climate and water potential. Such loss can be a dominant parameter in the water balance of arid or semi-arid areas. Classical soil physical and meteorological methods can be used to estimate evaporative fluxes over short time periods, but are difficult to use over the longer times which are appropriate for most water balance studies. We describe here a new method for the estimation of evaporation from a bare surface based on enrichment of natural deuterium. The advantage of this technique is that it provides an integrated measure over relatively long periods of time. Using this method on data collected from the bed of Lake Frome, a normally dry salt lake in Central Australia, we find that the evaporation rate from the 'dry' lake surface is 63 mm yr⁻¹.

Enrichment of the natural levels of deuterium and ¹⁸O in water occurs during evaporation. Change in the concentration of these isotopes in open water bodies has been used in attempts to obtain information about their water balance^{1–3}. The depth profile of deuterium concentration resulting from evaporation of water from a saturated sand column in the laboratory, was first measured by Zimmermann *et al.*⁴. Laboratory and theoretical studies on unsaturated materials have been reported recently^{5–8}, and the possibility of using the rate of decrease of the isotope concentration with depth to measure evaporation rate was suggested⁷. To our knowledge the present work represents the first attempt to apply this theory to estimating evaporation in the field.

The isotope profile that develops in a soil subject to evaporation is the result of a balance between the upward evaporative flux and the downward diffusive flux. In that part of the soil profile where movement in the liquid phase dominates, the relationship between the deuterium delta value, δ , and depth, z , is given by⁷

$$(\delta - \delta_{\text{res}}) = (\delta_{\text{ef}} - \delta_{\text{res}}) \exp(-f(z)/\hat{z}) \quad (1)$$

where δ_{res} and δ_{ef} are the delta values of soil water at depth in the soil and at the evaporating front, respectively; $f(z)$ is a modified depth function which takes into account the effect of varying water content (see ref. 7), and $\hat{z}$ is the decay length (or the mean penetration depth of Zimmermann *et al.*⁴) given as

$$\hat{z} = \bar{\theta} r D / E \quad (2)$$

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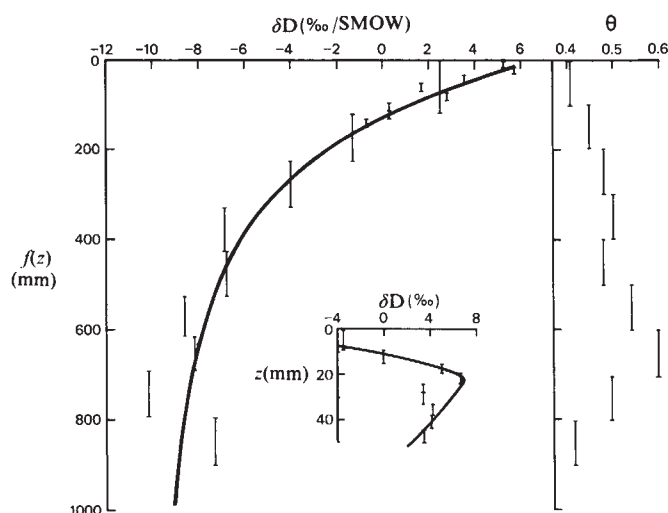


Fig. 1 The depth profiles of deuterium delta values, δ (‰/SMOW), and volumetric water content, θ , for Lake Frome sediments sampled 3 km from its western edge. Note that the ordinate is the depth function $f(z)$, rather than depth, z . Because θ is almost uniform, this has little effect on the shape of the profile. The inset shows details of the deuterium profile in the top 50 mm. The bars shown represent the depth intervals over which samples were collected. Samples were obtained by pushing a 50 mm diameter tube into the sediment and extruding it. Water samples from the sediments were obtained by azeotropic distillation with toluene. Errors in sample preparation dominate those of mass spectrometric analysis. The total error (1 s.d.) has been estimated as 2 δ units for the treatment used here¹⁴. Least-squares estimates of the parameters $\hat{z}$, δ_{res} and $(\delta_{\text{ef}} - \delta_{\text{res}})$ were obtained by an iterative procedure. The solid line represents the best fit to the data.

where $\bar{\theta}$ is the mean volumetric water content, τ is the tortuosity, D is the diffusion coefficient of HDO in liquid water and E is the evaporation rate.

Solution of equations (1) and (2) gives one method, usually the most reliable one, for estimation of evaporation rate. Two other methods⁷ rely on the existence of a region of vapour transport within the soil. These use either the depth of the evaporating front beneath the soil surface as indicated by the peak in the isotope profile, or the relationship between δ and depth in the region of vapour transport.

Lake Frome, the bed of which was sampled in this study, is a normally dry salt lake situated in northern South Australia at a latitude of 30.5°S. Mean annual rainfall is 100–125 mm and the lake rarely contains significant water—the last time being in 1973. In common with many other salt lakes in the arid region of Australia, the areal extent of the lake is determined by the intersection of the capillary fringe of the regional groundwater table with the soil surface (J. M. Bowler, personal communication). Thus, during the long periods between filling, lakes such as this are groundwater discharge points. The purpose of the study was to estimate the groundwater inflow to such lakes. A recent study⁹ has shown that groundwater inflow patterns to such lakes can be complicated by density stratification, but this will not be pursued here.

The depth profiles of water content and deuterium composition of the water contained in the sediments of the lake bed at a site ~3 km from the western edge of the lake are shown in Fig. 1. Using these data a least-squares value of 240 ± 30 mm was obtained for $\hat{z}$.

To obtain an estimate of evaporation rate from this value using equation (2), estimates of τ , θ and D are needed. For τ we used Penman's value¹⁰ of 0.66. Other work suggests that this value is adequate for materials which do not have a pore size distribution that is strongly bimodal⁸. We are aware that in materials having a high clay content, τ may be lower. A value of 0.45 was used for θ , and for D of HDO in saturated

NaCl solution at the measured sediment temperature of 30 °C, we used $1.45 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$ (ref. 11); from these values E is calculated at $63 \pm 8 \text{ mm yr}^{-1}$.

To estimate the evaporation rate from the depth of the maximum in the deuterium profile, z_{ef} , equation (3) is used as

$$z_{\text{ef}} = (h_{\text{ef}} - h_{\text{a}}) N_{\text{sat}} D_v \tau (p - \theta) / \rho E \quad (3)$$

where h_{ef} and h_{a} are the relative humidities at the evaporating front and of free air, respectively; N_{sat} is the saturated vapour concentration of air ($= 0.0304 \text{ kg m}^{-3}$ at 30 °C) and D_v is the diffusivity of water vapour in air ($26.5 \times 10^{-6} \text{ m}^2 \text{ s}^{-1}$ at 30 °C)¹²; p is total porosity of the sediments (0.55), and ρ is the density of water. Using the observed value of 22 mm for z_{ef} (inset, Fig. 1), the evaporation rate is calculated as 46 mm yr^{-1} . It is difficult to assess the error associated with this estimate, but the relative standard deviation is probably ~25%.

It has been established⁷ that evaporation rate can also be estimated from the slope of the relationship between a function of δ and the inverse of depth for the region above the maximum in the deuterium profile. However, this method is subject to considerable error as few data are available and small discrepancies in depth, especially near the surface, will have a large effect on the estimate. Ignoring the surface δ value, E is calculated as 35 mm yr^{-1} . Again error is difficult to assess, but the relative standard deviation is probably ~50%.

Of the three estimates of evaporation rate of 63, 46 and 35 mm yr^{-1} , as mentioned earlier, the first should be the most reliable, while the third is by far the least reliable.

Given the high volumetric water contents (> 0.4) near the surface it is surprising that a vapour transport layer develops. We assume that its existence is due to pore water being saturated with NaCl at the surface and less than saturated at depth. This gives rise to a vapour pressure gradient and hence allows the possibility of vapour movement.

The characteristic time ($D\bar{\theta}\tau/E^2$) for development of the isotope profile described here is several years. This has two important implications: first, the estimate of evaporation represents the mean over a considerable period of time, and second, the technique is particularly suited to arid areas where rainfall and flooding distort the isotope profile only rarely. However, this technique should also be useful in areas which are seasonally arid. In such areas the evaporation rate will usually be greater and the deuterium profile will reach steady-state conditions more quickly, but extend to a lesser depth.

A very approximate estimate of potential evaporation from a saturated soil surface in the vicinity of the lake is $\sim 2,200 \text{ mm yr}^{-1}$ ($0.7 \times 3,200$, the evaporation from a class A pan¹³). The difference between this and the value of 63 mm yr^{-1} obtained for evaporation from the salt-encrusted surface is surprising at first sight, as the water table is at a depth of 300 mm and the capillary fringe rises to the surface. However, actual evaporation is reduced below potential by two factors. (1) The vapour pressure deficit between the atmosphere and the water at the evaporating surface is reduced because this water is saturated with NaCl. (2) The surface of the lake bed is near white and the albedo will be much higher than that for a free water surface. The estimate of evaporation rate is consistent with that of 50 mm yr^{-1} obtained from an approximate hydrological balance for Lake Torrens, a nearby and similar lake (J. W. Holmes, personal communication).

Dr J. M. Bowler raised the problem of evaporation from dry lake surfaces and provided logistic support during sampling. Drs E. L. Greacen and W. J. Stone helped during sampling and in discussion and Mr M. W. Hughes assisted with the analyses and took part in useful discussions. C.J.B. was supported by a grant from the Engineering and Water Supply Dept, South Australia.

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Latitudinal displacement from main moisture source controls $\delta^{18}\text{O}$ of snow in coastal Antarctica

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$\delta^{18}\text{O}$ in polar precipitation is usually correlated with the condensation temperature^{1,2}. Here, observations of the oxygen isotopic composition of coastal Antarctic precipitation at Syowa Station^{3,4} during 1974 are re-evaluated, and it is demonstrated that monthly average $\delta^{18}\text{O}$ is more closely associated with the preceding month's mean temperature than that at the time of sampling. Linear regression indicates that the lag between temperature and isotopic ratio occurs because sea ice extent is a dominant factor for $\delta^{18}\text{O}$ values in Antarctic precipitation (Fig. 1). The annual growth and decay of Antarctic sea ice parallels the migration of the primary moisture source region which is located in the vicinity of the 0 or 1 °C sea surface isotherm. We conclude that the meridional distance from the primary moisture source is the main determinant of $\delta^{18}\text{O}$ values in coastal Antarctic precipitation.

Although it is widely known that long-term climatic information is recorded in the world's ice caps by the oxygen isotopic composition of past precipitation, relatively little effort has been devoted to understanding the atmospheric input signal. Koerner⁵ demonstrated that $\delta^{18}\text{O}$ values from the Queen Elizabeth Islands in Arctic Canada are linked with both the distance to the nearest appreciable moisture source and the elevation of sampling. The first factor appears to conflict with the well established multi-year relationship⁶ between $\delta^{18}\text{O}$ and temperature. Robin⁷ suggested that this contradiction can be resolved for Antarctica if temperature and displacement from the water vapour source are related. By using isotopic data^{3,4} from the coastal location at Syowa, where orographic influences are small, the relationships between $\delta^{18}\text{O}$ and both variables have been analysed and the results were found to confirm Robin's hypothesis.

The 1974 record of the $\delta^{18}\text{O}$ values in Syowa precipitation was investigated quantitatively by applying linear regression analysis on a monthly time scale. Of the 38 $\delta^{18}\text{O}$ measurements, 16 included some drift snow; such contamination does not appear to mask the $\delta^{18}\text{O}$ signal in precipitation⁴. For monthly average variables ($n = 11$), linear correlation coefficients of 0.6 ($r^2 = 0.36$) are significantly different from zero at the 95% confidence level.

The previous qualitative analysis of these data⁴ suggested that temperature of condensation (which theoretically is related to $\delta^{18}\text{O}$) is not the main factor controlling $\delta^{18}\text{O}$. Table 1 defines the three types of monthly temperature variables used in the present study: general (representing all conditions), precipitation, and δ -sampled. Figure 2 shows the erratic nature of $\hat{T}_p^{\delta^{18}\text{O}}$ compared with the smooth variation of $\delta^{18}\text{O}$ (except for August) and the other two temperature variables. This erratic

behaviour, which is shown by both the $\delta^{18}\text{O}$ -sampled surface and cloud temperature variables, means that they explain only a small percentage of the variance of $\delta^{18}\text{O}$ (see concurrent r^2 values in Table 1). This is in contrast to the higher degree of association between $\delta^{18}\text{O}$ and the concurrent precipitation and general temperature variables. Cloud temperatures approximate the condensation temperature rather than surface values, and thus correlate better with $\delta^{18}\text{O}$. However, because both the $\delta^{18}\text{O}$ sampled temperature variables have a weak association with $\delta^{18}\text{O}$, the temperature of condensation does not seem to be the principal determinant of $\delta^{18}\text{O}$.

Kato⁸ also found that the preceding month's mean temperature was more closely associated with $\delta^{18}\text{O}$ than the concurrent temperature. Comparison of both columns of the coefficients of determination (r^2) in Table 1 strongly support this observation. Note that the general temperature variables yield the best association with $\delta^{18}\text{O}$ when regression with the previous month's temperature is considered. In fact, any general temperature of the previous month taken at any standard level below 600 mbar is strongly associated with $\delta^{18}\text{O}$. Thus, $\delta^{18}\text{O}$ is more closely related to the atmospheric heat budget of the previous month as opposed to the temperature at the time of precipitation. This circumstance is masked when $\delta^{18}\text{O}$ and T_s are averaged over many years, and thus is not in conflict with the multiannual $\delta^{18}\text{O}$ -temperature relationship⁶ established for the present climate.

Sea ice growth to the north of Antarctica is delayed relative to air temperature by about a month and has been inferred as the cause for the higher $\delta^{18}\text{O}$ values between December and May in comparison to those for July–November⁴. Antarctic sea ice areas for 1974 have been derived from the passive microwave observations collected by the ESMR instrument onboard the NIMBUS 5 satellite⁹. Mid-month areas of sea ice between the longitudes 60° W and 90° E with ice concentrations of at least 15% and at least 85% were regressed with $\delta^{18}\text{O}$. Although results are independent of the longitudinal sector of sea ice considered (for example, the entire area of Antarctic sea ice yields similar results), the area with 15% or higher ice concentration explains 79% of the variance of $\delta^{18}\text{O}$ when the variables are in phase. When the areas of highly concentrated

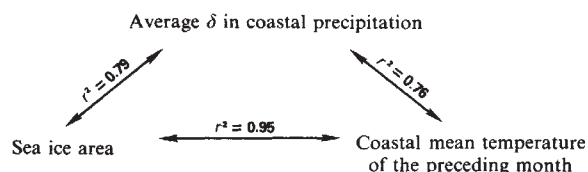


Fig. 1 Interrelationships between $\delta^{18}\text{O}$ (abbreviated as δ) in coastal precipitation, sea ice area and coastal mean temperature.

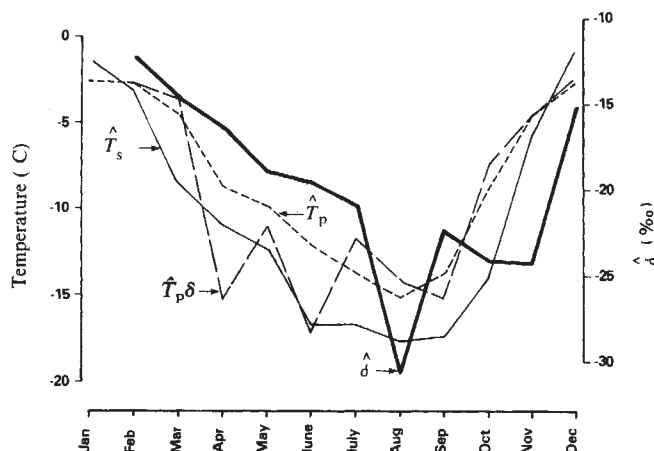


Fig. 2 Variations in $\hat{T}_s$, $\hat{T}_p$, $\hat{T}_p^{\delta^{18}\text{O}}$ and $\delta^{18}\text{O}$ during 1974 at Syowa Station.

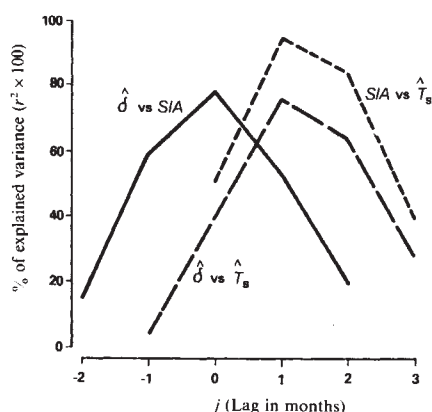


Fig. 3 Coefficients of determination (r^2) resulting from regressions:

- (a) $(\delta^{18}\text{O})^{i+j} = A + B(\hat{T}_s)^i$
 (b) $(\delta^{18}\text{O})^{i+j} = A + B(\text{SIA})^i$
 (c) $(\text{SIA})^{i+j} = A + B(\hat{T}_s)^i$

where i indicates the month and j specifies the lag in months in each of the regression equations. B is the regression derived slope of the line and A is the intercept. SIA is the area of sea ice with concentration $\geq 15\%$. An example of a positive lag ($j = 1$) is $\delta^{18}\text{O}$ for March compared with $\hat{T}_s$ for February.

ice ($\geq 85\%$) are considered $\delta^{18}\text{O}$ and sea ice area variations are no longer in phase. This indicates that the origin of moisture being precipitated at Syowa is closer to the 15% ice concentration isopleth.

The interrelationships between sea ice area, mean temperature and $\delta^{18}\text{O}$ are summarized by Fig. 1. It is inferred that the physical control of $\delta^{18}\text{O}$ is related to the area of sea ice because their variations are in phase. The $\delta^{18}\text{O}$ -temperature correlation arises because temperature governs sea ice growth ($r^2 = 0.95$ when $\hat{T}_s$ leads by 1 month the area of 15% or greater

ice concentration); the approximate 1 month lag between temperature forcing and sea ice response must be a consequence of the large thermal capacity of the ocean. A similar relationship between zonal averages of the latitude of the sea ice edge and the temperature was noted by Cavalieri and Parkinson¹⁰. Figure 3 depicts the change in the correlations between the variables as a function of lag, and demonstrates that the maximum percentage of explained variance is achieved by the relationships given in Fig. 1.

Additional support exists for the associations given in Fig. 1. The same strong lagged relationship between sea ice area and mean temperature at Syowa applies for 29 of the 36 months between 1973 and 1975 (7 months of sea ice data are missing). The $\delta^{18}\text{O}$ -lagged $\hat{T}_s$ relationship holds for coastal Roi Baudouin^{2,11}; $\delta^{18}\text{O}$ values collected at Mizuho Camp (elevation 2,230 m and 270 km inland of Syowa) for part of 1974 also exhibit this behaviour¹². However, examination¹³ of the South Pole $\delta^{18}\text{O}$ data set¹ showed no evidence for a lag between $\delta^{18}\text{O}$ and temperature on a monthly time scale. The multiannual $\delta^{18}\text{O}$ -temperature diagram given by Dansgaard *et al.*¹⁴ also shows that the coastal areas and interior parts of Antarctica behave quite differently. Further research is needed to identify the physical basis of this apparent difference.

Robin⁷ suggested on the basis of Koerner's⁵ result for the Canadian Arctic that the sea ice effect found by Kato⁴ could be explained if the water vapour source region were represented by the position of the sea surface isotherms north of the pack ice limit; the most southerly isotherms undergo an annual migration in phase with that of the pack ice. Thus, the hypothesis is that the annual $\delta^{18}\text{O}$ variation in coastal precipitation is due to variations in the meridional distance between the source region and the precipitation site. In the immediate vicinity of Syowa Station, the area of sea ice within a given longitudinal sector is very nearly proportional to the meridional distance between the zonally averaged latitude of the sea ice edge and Syowa. Therefore, the present analysis indicates that $\delta^{18}\text{O}$ of

Table 1 Coefficients of determination (r^2) resulting from regression of monthly mean temperature variables[†] with $\delta^{18}\text{O}$ ($n = 11$) for Syowa Station in 1974

Variable		Definition	Concurrent r^2	Temperature leads by 1 month r^2
$\delta^{18}\text{O}$		Average of $\delta^{18}\text{O}$ values weighted by the estimated snowfall amount in each event	—	—
General temperatures	$\hat{T}_{850}$	Monthly mean temperature at 850 mbar level	0.40	0.77
	$\hat{T}_s$	Monthly mean temperature at surface	0.40	0.76
Precipitation temperatures	$\hat{T}_{cl}$	Monthly mean cloud temperature* during precipitation	0.58	0.64
	$\hat{T}_p$	Monthly mean surface temperature during precipitation	0.45	0.72
$\delta^{18}\text{O}$ -sampled precipitation temperatures	$\hat{T}_{cl}^{\delta^{18}\text{O}}$	Same as above $\hat{T}_{cl}$ but only including temperatures during precipitation sampled for $\delta^{18}\text{O}$	0.20	—
	$\hat{T}_p^{\delta^{18}\text{O}}$	Same as above $\hat{T}_p$ but only including temperatures during precipitation sampled for $\delta^{18}\text{O}$	0.19	—

[†] General temperatures are simple averages. The remaining four temperature averages (as well as $\delta^{18}\text{O}$) are weighted by the estimated snowfall amount of each precipitation event. The caret denotes a 1-month time average. Data are tabulated in ref. 17.

* Obtained by vertically averaging the temperature readings between the cloud boundaries (defined by saturation with respect to liquid water); weighting is with respect to water vapour mixing ratio.

Table 2 Regression analysis between $\delta^{18}\text{O}$ for Syowa in 1974* and the latitudinal distance (for the same month) from Syowa to the sea ice edge and to various sea surface isotherms ($n = 11$)

Monthly average independent variable [†] (km)	Ref.	Slope ± 1 s.e. (% km ⁻¹)	Intercept ± 1 s.e. (%)	r^2
Meridional distance to sea ice edge in 1974	18	-0.009 ± 0.001	-13 ± 1	0.82
Meridional distance to average sea ice edge 1972–77	19	-0.009 ± 0.002	-12 ± 1	0.80
Meridional distance to 0 °C isotherm‡	20	-0.007 ± 0.001	-10 ± 2	0.74
Meridional distance to 1 °C isotherm‡	20	-0.008 ± 0.001	-7 ± 2	0.75
Meridional distance to 2 °C isotherm‡	20	-0.014 ± 0.002	$+7 \pm 5$	0.79

* The anomalous observed $\delta^{18}\text{O}$ value for August was replaced by the average of the July and September values.

[†] Zonally averaged between 20° and 60°E. Meridional distance is defined to be positive when measured northwards from Syowa.

‡ Long-term average isotherm locations based on all available ship observations.

Syowa precipitation is in phase with and is linearly proportional to this distance.

To isolate the location of the water vapor source, the $\delta^{18}\text{O}$ record for Syowa was compared with the average for the same month of the meridional distance between Syowa and the following features: the pack ice edge and the 0, 1 and 2 °C sea surface isotherms. The results of this analysis are set out in Table 2. Unfortunately, only long-term average positions of the isotherms were available. The very similar results obtained when $\delta^{18}\text{O}$ was regressed against both the 1974 and 1972–77 sea ice edges suggest that the $\delta^{18}\text{O}$ versus 1974 isotherm comparison would not differ significantly from the isotope–isotherm analysis given in Table 2. A latitudinal isotopic gradient of $\sim 0.009\text{‰ km}^{-1}$ is compatible with the atmospheric water balance at Syowa Station (work in preparation). The much larger gradient obtained by Koerner⁵ of 0.02‰ km^{-1} may arise because the orography of the Queen Elizabeth Islands causes

more depletion of vapour¹⁵ (hence a more rapid decrease in $\delta^{18}\text{O}$) per km compared with the nonorographically induced precipitation over the pack ice upwind of Syowa. The intercept represents the $\delta^{18}\text{O}$ value of oceanic precipitation, which is implicitly assumed to be constant throughout the year. Aldaz and Deutsch¹ and Dansgaard⁶ pointed out that this may be -8‰ for latitudes in the vicinity of 50° S. We thus conclude that the primary origin of moisture being precipitated at Syowa lies in the vicinity of the 0 or 1 °C isotherm. On an annual basis, the corresponding average latitudes are 58° and 55° S, respectively; these source regions are poleward of those proposed previously^{1,16}. Accurate, simultaneous data probably will be needed to clarify the situation.

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Reconstruction of Palaeogene climate from palynological evidence

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Multivariate statistical analysis of Palaeogene (early Tertiary) pollen and spore spectra from north-west Europe allows the identification of four major groups of taxa¹. Three are thought to include the Palaeogene equivalents of taxa now found in deciduous forest, fern and conifer forest and paratropical rain forest. The other is a rubbish-bin of plant taxa reflecting the influences of transport and catchment processes. We have disentangled these effects from the ecological processes and, by analogy with plant megafossil evidence, obtained estimates of summer maximum and winter minimum temperatures, as well as estimates of mean annual temperatures. The resulting picture of climatic fluctuations in the Palaeogene can be recognized in localities throughout north-west Europe. We show here that the general similarity to results of oxygen isotope analyses from deep-sea cores² and the North Sea³ suggests that genuine global climatic changes are being detected. Major differences between 'Oligocene' and 'Eocene' climates are also suggested by these reconstructions and support conclusions from other evidence⁴.

At the heart of our study were detailed quantitative analyses of 187 samples containing 85 palynological form-genera from three boreholes (Ramnor, Bunker's Hill and Shamblehurst Lane) in the Hampshire Basin of southern England. These span most of the Eocene from the bottom of the London Clay to near the top of the Headon Beds⁵. The boreholes are in what is believed to have been mainly a shallow-water marine area, perhaps 10–20 km from the coast. A wide range of sedimentary environments appear to have been sampled—marine, brackish, estuarine and lagoonal. 'Principal components analysis' of the pollen percentages identified 31 non-trivial associations of taxa of which 20 (after variable-maximizing rotation and cluster analysis of the original correlation matrix) could be readily interpreted in ecological terms, while the remaining 11 were enigmatic or described groups of pollen taxa that were clearly

associated with catchment regimes rather than ecology⁶. For instance, the association of *Graminidites*, *Milfordia* and *Spar-ganiaceapollenites* in the same principal component is strongly suggestive of a marsh community, and because some of the other associated taxa have been compared with tropical forest plants, an analogy to swamps found in tropical river deltas today can be suggested. The relative clarity of the distinction between catchment-related and ecologically informative principle components may be a result of the length of the chronological period considered, or of the heterogeneity of the sediments sampled, or a combination of both features.

Our recent study of these 20 ecologically informative rotated principal components suggested that they could be sorted into three major taxonomic groups having ecological significance: deciduous forest, fern and conifer forest and paratropical rain forest¹. Figure 1 illustrates the dendrogram produced by the average-method clustering of the original correlation matrix. It indicates the gross structure of the matrix, and reveals similar groupings to those defined by principal components analysis. The composition of the three groups invited comparison with Wolfe's leaf physiognomic classifications of extant⁷ and extinct⁸ floras, and are consistent with the relationships to modern plant families proposed by Muller⁹. The stratigraphical distribution of these three different groups of principal components is reflected in the pollen diagrams of Fig. 2. The principal components of group III (paratropical rain forest affinity) appear to be of major importance in the uppermost London Clay and basal Headon Beds, while above this, principal components of group I pollen (related to modern deciduous forests) play a major part.

Similar calculations have now been made for other palynological data from the Oligocene of the western British Isles^{10,11}, and the Tertiary of the Paris Basin¹² and the Armorican Massif¹³. The results suggest that our equations are valid and applicable far outside the geographical and chronological range for which they were designed. These results are shown in Fig. 2 which represents the first objective and quantitative palynological historical record of Palaeogene Europe.

The traditional methods of palaeopalynological biostratigraphical correlation depend on the presence or absence of pollen types having stratigraphically limited ranges. There are 28 pollen taxa known to have restricted chronological distributions in the Eocene of southern Britain¹⁴, by means of which

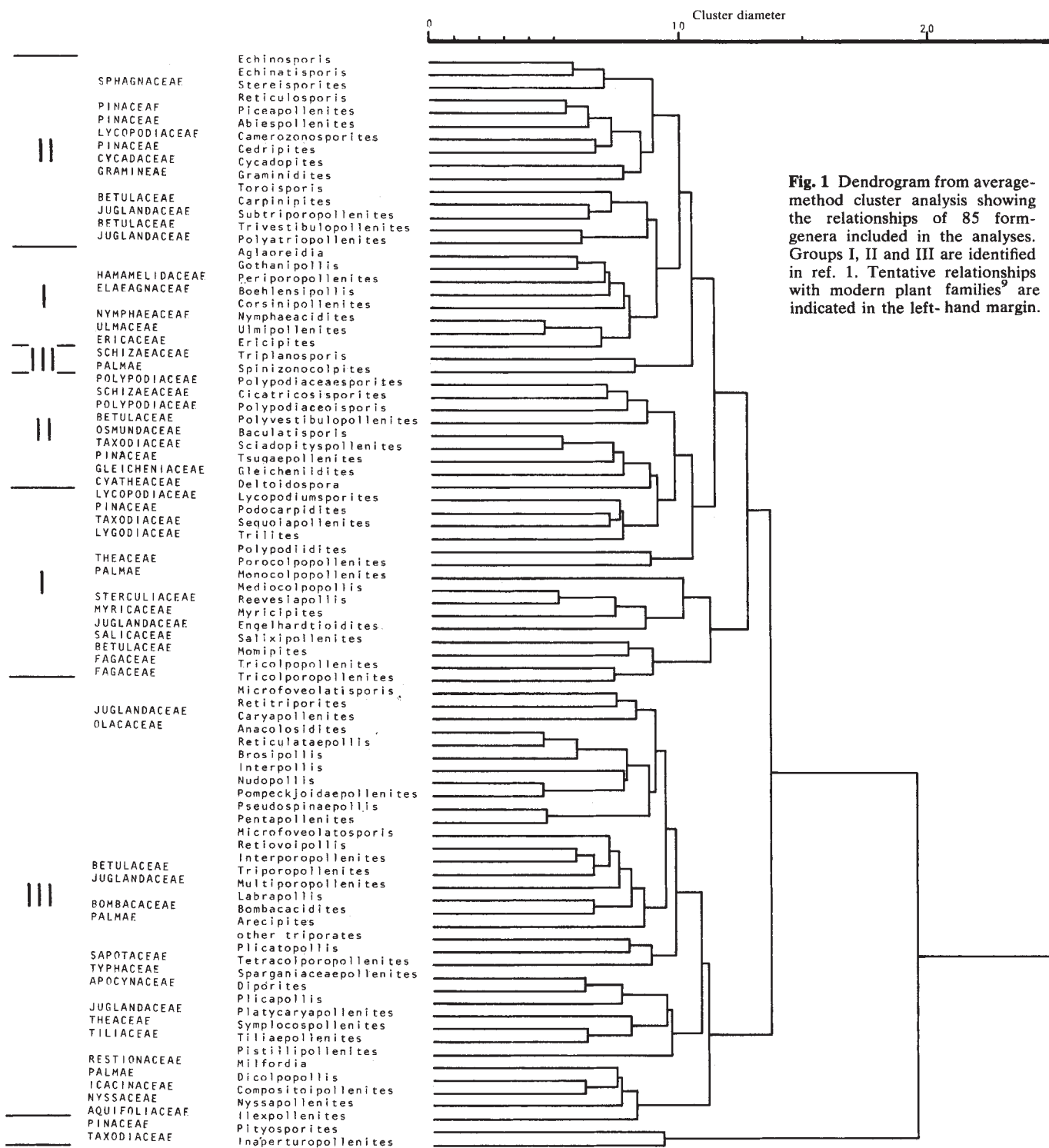


Fig. 1 Dendrogram from average-method cluster analysis showing the relationships of 85 form-genera included in the analyses. Groups I, II and III are identified in ref. 1. Tentative relationships with modern plant families⁹ are indicated in the left-hand margin.

the Ramnor borehole could be related to other sequences, but which did not permit the satisfactory correlation of the Bunker's Hill and Shamblehurst Lane analyses. The correlations in Fig. 2 are much more accurate and of greater reliability and applicability than the ones based on range-fossils. Correlations such as those between the Lundy borehole and the Corneilles section, and between the freshwater lacustrine Bovey Basin deposits and the marine sediments of the Lundy and Corneilles sequences demonstrate the efficacy of the discrimination between catchment-related effects and real ecological signals. In fact, of 21 sections studied, only 3 could not be satisfactorily correlated, mainly because they were too short.

If our comparisons of the major palynological groupings with the equivalent megafossil evidence are correct, the climatic

parameters associated with the leaf-physiognomic groupings can then be associated with the changing palynological assemblages to produce a crude estimate of the climatic changes between about 25 and 60 Myr ago. We have associated the following climatic conditions with the major palynological groupings by taking representative parameters from Wolfe's⁷ data (Table 1).

The results are plotted in Fig. 3 in terms of a predicted mean annual temperature and associated summer maxima and winter minima.

Since Wolfe has pointed out that the results of evolutionary processes mean that climatic and ecological associations of the past cannot generally be related to modern taxonomic groupings, our climatic assumptions might appear unjustifiable. The

Table 1 Association of climatic conditions with major palynological groupings

	Mean annual temperature (°C)	Mean annual temperature range (°C)
Deciduous forest	10.0	30.0
Fern and conifer forest	14.0	14.0
Paratropical rain forest	22.5	8.0

relationship between pollen morphology, taxonomy, leaf physiognomy and ecology is, at best, devious; and not such as to encourage detailed comparisons. Nonetheless, the degree of correlation between the hard facts of climatology revealed by isotopic analyses, and the consequences of our necessary assumptions demonstrate that in certain family and generic groupings there has been no gross overall change in climatic and ecological preferences.

Figures 2 and 3 show that a pronounced and prolonged warming episode occurred in the top of the London Clay and

bottom of the Bracklesham Beds (Zone b), and that a second equally pronounced, if more short lived, event occurred at the bottom of the Headon Beds (Zone e). A further brief torrid phase seems to have taken place in the Oligocene and is recorded at Bovey Tracey, Mochras and Lundy. The periods preceding and succeeding the London Clay–Bracklesham Beds thermal maximum (palynological Zones a and c) seem to have been cooler than the intervening Zone b, and had a generally unstable, if equable, climate. Above Zone c, a distinct decrease in climatic equability can be recognized (Zone d) in which the tropical Zone e may only be a substage. In contrast, the Oligocene climate seems to have been uniformly cooler and relatively stable, but marked by strong fluctuations in the severity of the winters, which were often of pronounced fridity. Although patterns of botanical fluctuations occur throughout this period, only two episodes at present seem to merit recognition as distinct zones. Zone v is apparently terminated by a period of more equable, cool climate (Zone w) while Zone v itself is punctuated by the brief, warm v₂ episode already mentioned, which is a valuable stratigraphical marker horizon. In the absence of any precise stratigraphical relationship between these Oligocene deposits it is difficult to relate them accurately

Fig. 2 Summary of botanical developments during the European Palaeogene as recorded by pollen analysis. In each diagram the lower ruled part represents deciduous forest elements (group I), the lower blank area represents fern and conifer forest components (group II) and the upper ruled area refers to the paratropical rain forest component (group III). The upper blank area is for pollen taxa of ambiguous or uncertain affinities. It is impossible to plot more than about 80% of the pollen spectra in most of the sequences illustrated. The time scale is by analogy with Shackleton and Boersma². Depth scales, where available, are marked at 50-m intervals. The Hamstead section is only 8 m deep.

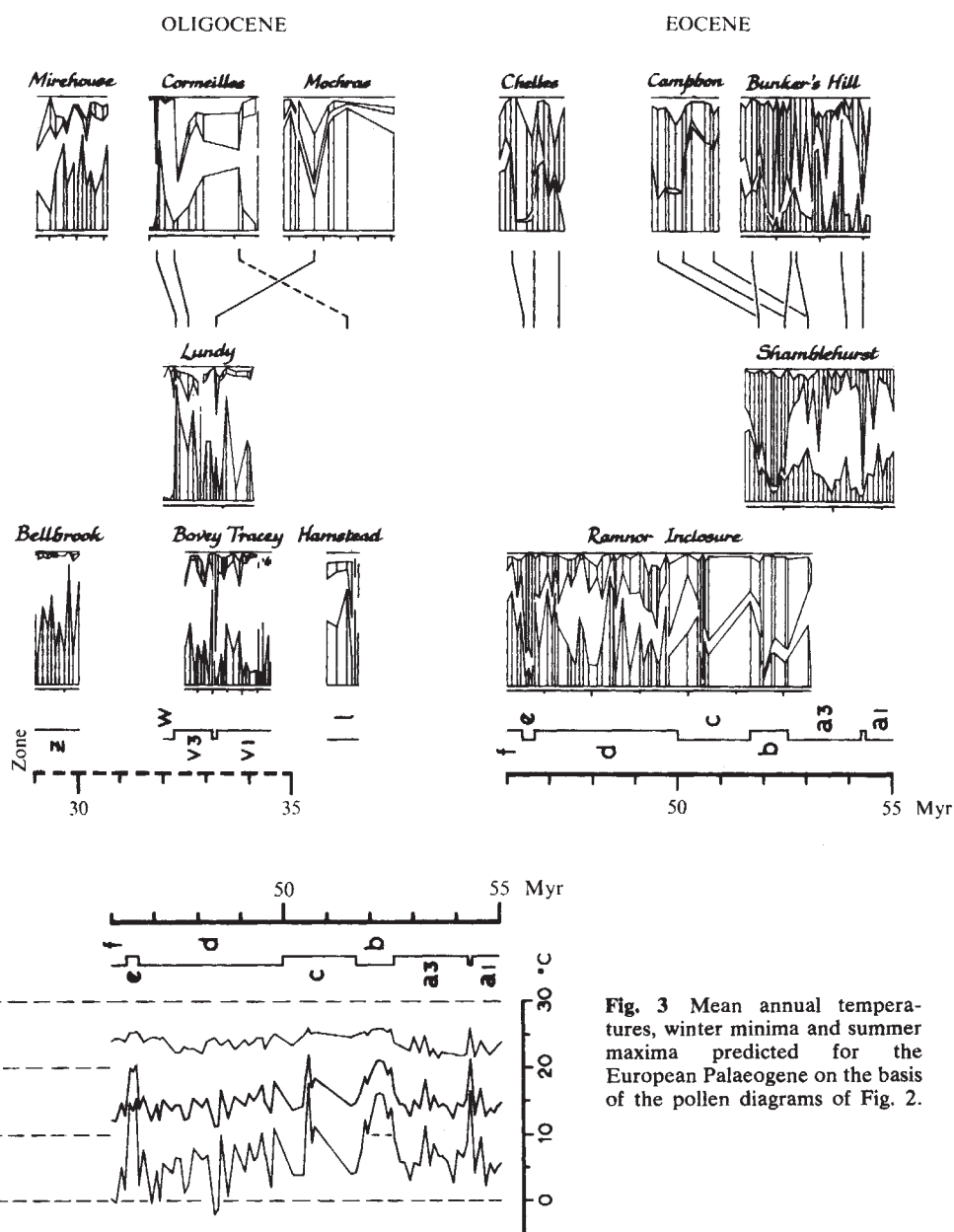


Fig. 3 Mean annual temperatures, winter minima and summer maxima predicted for the European Palaeogene on the basis of the pollen diagrams of Fig. 2.

to the deep-sea results, and the correlations proffered here are tentative.

The climatic and environmental interpretations offered here are also of palaeogeographical and phytogeographical interest. The brief and marked warming episode indicated by the pollen from cores of the Mochras borehole (on the Cardigan Bay coast of North Wales) allows correlation with the Lundy and Bovey Tracey boreholes. In comparison with these localities, however, the mean annual temperatures at Mochras are 2–3 °C lower, deciduous forest components are more abundant and the fern and conifer forest element is rarer. We believe that these discrepancies provide evidence for the existence of higher land in North Wales during the Oligocene. Similarly, the Calcaire de Campbon section in the Armorica Massif can be correlated with the thermal maximum of the uppermost London Clay–lower Bracklesham Beds; but the deciduous elements are again disproportionately well represented and the fern and conifer forest components are almost entirely absent. These interpretations could imply that the 'montane' flora of Tertiary Europe was essentially temperate and deciduous in character and that the fern and conifer forest was essentially an inland lowland flora.

It is instructive to compare the botanically-based climatic predictions with the absolute climatic determinations from $^{16}\text{O}/^{18}\text{O}$ isotope ratios in foraminiferal tests². The major trends of the Eocene are clearly recognizable in both records, especially Zone b between 51 and 53 Myr. We identify Zone e with the isolated warm point 47 Myr ago, and Zone v₂ (very tentatively) with a similarly isolated warm point at about 33 Myr. Our predictions of mean annual temperature in Zone a are about 4 °C higher than Shackleton's determinations, in Zone f about 3 °C higher, in Zone v₂ about 13 °C higher, and elsewhere about 6 °C higher. As no absolute method of determining seasonality in the Tertiary is available, these predictions cannot be checked. In part, the discrepancies probably arise from misjudgement of the climatic parameters suggested by Wolfe's work, in part from misinterpretation of structures still to be discovered in Tertiary vegetation, partly from evolutionary differences between modern floras and their Tertiary analogues, and probably mainly from the notoriously nonlinear relationship between pollen spectra and vegetation. Despite these discrepancies, our results suggest a fundamental climatic difference between Oligocene and Eocene environments: the Oligocene seems to have been characterized by a climate whose maximum summer temperatures and mean annual temperatures were fairly constant, but whose winters were of varying but often considerable severity, while in the Eocene the winters were generally mild, and the summer and mean annual temperatures changed considerably. On these grounds the Oligocene/Eocene boundary would have to be placed somewhere above the top of the Ramnor borehole. On the other hand, it could be argued that the fundamental difference lay in the overall warmth. On this criterion the boundary would be put at the junction of Zones c–d, where a persistent cooling first becomes apparent.

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Order in the initial retinotectal map in *Xenopus*: a new technique for labelling growing nerve fibres

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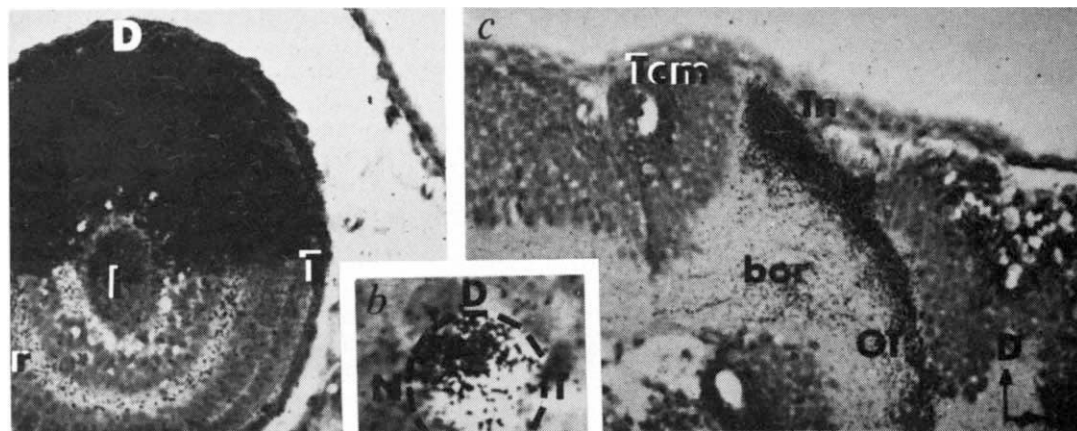
Retinal nerve fibres form an orderly map of visual space in several centres in the vertebrate brain^{1,2}. Such topographic maps are a common feature of central nervous system organization^{3–5}, yet the way in which they develop is poorly understood. Early nerve projections in the fetal and neonatal mammalian brain have been found in several cases to be less restricted than those in the adult^{6–9}, suggesting that nerve fibres may initially form a diffuse set of connections in their target structure from which the adult map is sculpted by the elimination of terminals. Indeed, previous electrophysiological data indicate that the retinotectal map in *Xenopus laevis* might be initially disorganized¹⁰. We report here, however, that the retinotectal projection is ordered from the beginning of tectal innervation (stage 39/40). We demonstrate this first autoradiographically by tracing groups of growing ganglion cell axons which we labelled by incubating sectors of eye rudiments, before axonal outgrowth, in ³H-proline and replacing them orthotopically. Separate labelling of dorsal and ventral parts of the initial projection showed that retinal fibres are organized topographically, as in the adult¹¹, in the tectal rudiment and throughout much of the pathway. Second, we show that visual responses are ordered in the tectum from the first stage that they can be mapped (stage 40). We conclude that the topographic ordering of retinotectal connections develops as a result of directed axonal outgrowth.

Questions of how nerve connections are formed between the retina and the tectum have commonly been approached by studying regenerating optic projections^{12–16}. In lower vertebrates the retina can regenerate a normal visual map in the tectum and electrophysiological^{13,14} as well as recent anatomical data^{15,16} have indicated that the topography becomes re-established in a stepwise fashion. The investigations of the primary outgrowth of axons in the vertebrate central nervous system have been hindered because, possibly due to the high content of extracellular space in the embryonic neuroepithelium, nerve tracers such as horseradish peroxidase and ³H-proline tend to spread extensively from the site of injection in young embryos¹⁷ (although see ref. 18). We have overcome this difficulty by pre-labelling sectors of presumptive eye tissue *in vitro* which enabled us to examine the topography of the first axonal projection from the eye to the tectum.

Grant and co-workers¹⁹ have shown that optic axons first leave the eye at stage 28 (staging according to ref. 20), about 33 h post-fertilization. Pre-labelling experiments were performed before axonal outgrowth, at optic vesicle stages 22–26, on embryos anaesthetized in 66% Niu Twitty solution²¹ containing 1:10,000 tricaine methane sulphonate (MS222, Sandoz). Whole eye primordia ($n=41$) or dorsal ($n=45$) and ventral ($n=43$) sectors were excised and incubated for 20–30 min in a drop of ³H-proline (specific activity 117 Ci mmol⁻¹; Amersham) in 66% Niu Twitty solution (1 mCi ml⁻¹). After incubation, each piece of eye tissue was washed six times and replaced in the donor embryo in its original position and orientation. Because the optic stalk gives rise to the ventral retina²², care was taken to include this structure when labelling the ventral eye primordium. Young larvae were fixed at stages 34–47 in 2.5% glutaraldehyde in 1% sucrose-phosphate buffer (0.1 M, pH 7.4) for 1.5 h, embedded in paraffin and sectioned

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Fig. 1 Autoradiographs showing label in the eye (a), the optic nerve head (b) and the midbrain (c) following ^3H -proline incubation of dorsal (a, b) and ventral (c) parts of eye primordia. a, Transverse section of a stage 43 retina; autoradiographic grains (black) are confined to the dorsal half corresponding to the region of incubation. Grains extend $\sim 60\ \mu\text{m}$ into the unlabelled ventral half in the inner plexiform layer, suggesting that dendritic processes are labelled. b, Transverse section of optic nerve head at stage 40; grains are localized to the dorsal half, indicating that optic axons leave the eye in retinotopic order. c, Parasagittal section of a stage 40 midbrain showing the labelled projection from the ventral half of the retina in the tectal neuropil (Tn), the dorsal edge of the optic tract (OT) and the basal optic root (bor). pe, Pigment epithelium; r, retina; l, lens; Tcm, tectal cell mass; D, dorsal; V, ventral; N, nasal; T, temporal; R, rostral. Scale bars: a, $100\ \mu\text{m}$; b, $10\ \mu\text{m}$; c, $50\ \mu\text{m}$.



at 6 or $10\ \mu\text{m}$. Sections were coated with Kodak K5 nuclear gel emulsion and exposed at 4°C for 2–4 weeks. Autoradiographs were developed in Kodak D19 and counterstained in modified Harris's haematoxylin.

Recordings were made from the tecta of 18 animals from stages 39 to 47, two at each stage. The mapping procedure, using a 20° black ball, illustrated in Fig. 3, is described in the legend. To eliminate subjective bias, experiments were performed blind; the experimenter who mapped the visual responses did not know where the other experimenter had positioned the electrode. The small size of the tectal neuropil ($50 \times 90\ \mu\text{m}$ at stage 40) permitted only two or three electrode placements to be made per animal at early stage 40s. We moved the electrode along the longest, rostrocaudal, dimension and thus mapped only in the nasotemporal direction. This axis was not examined autoradiographically because nasal fibres of passage, which traverse the rostral tectum to reach caudal sites, would not be distinguishable from the temporal fibres that terminate in the rostral region.

Experimental eyes appeared normal in $\sim 60\%$ of the cases; the other 40% showed signs of surgical trauma and were discarded. Autoradiographs of experimental retinas showed that label was confined to the cells that had been incubated in ^3H -proline (see Fig. 1a). The labelling pattern in the optic nerve head paralleled that in the retina (see Fig. 1b), indicating that the first population of axons leave the eye in a retinotopic fashion. This order was maintained in the distal part of the optic nerve close to the eye but could not be detected near to or at the optic chiasm. This apparent loss of order might result from a pathway reorganization of the sort needed to marshal fibres into a new order appropriate for entering the brain^{11,23}.

Incubations of whole eye primordia showed that optic fibres first reach the tectum at stage 38/39, which agrees with previous reports¹⁰. These are fibres from the dorsal retina, as partial eye incubations demonstrated that fibres from the ventral retina do not arrive until stage 40. Label in the brain at these and earlier stages was confined to normal visual pathways.

Separate labelling of dorsal or ventral parts of the projection showed that topographic order is present in the tectum and optic tract from the beginning of tectal innervation (stage 39 onwards). This is illustrated in Figs 1c and 2. Like the adult, fibres from the ventral retina project to the dorsomedial tectum (see Fig. 2a) and lie dorsally in the optic tract (Fig. 1c). Those from the dorsal retina terminate in the ventrolateral tectum (Fig. 2b) and occupy more ventral positions in the tract (not shown).

Witkovsky *et al.*²⁴ have shown that when phototransduction begins in the *Xenopus* retina (stage 39), the stimulus threshold is initially high, but then falls rapidly (stages 39–43). We first recorded visual responses from the tectum at stage 39. These,

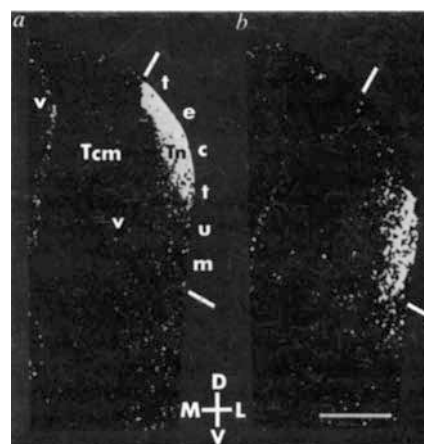


Fig. 2 Dark field autoradiographs showing the topographic distribution of the first optic afferents to arrive in the tectum. Transverse sections of the mid-tectum at stages 40 (a) and 39 (b); axons from the labelled ventral retina terminate dorsomedially (a) whereas dorsal retinal fibres terminate ventrolaterally (b). Tn, tectal neuropil; Tcm, tectal cell mass; v, tectal ventricle; D, dorsal; V, ventral; M, medial; L, lateral. Scale bar, $30\ \mu\text{m}$.

elicited by direct flashes of intense illumination ($9.5 \times 10^4\ \text{erg cm}^{-2}\ \text{s}^{-1}$), were weak and could not be driven by the black ball stimulus. From stage 40, however, the responses were stronger and could be mapped. The responses were multi-unit, with large receptive fields, usually $30\text{--}90^\circ$. In each case ($n = 16$) a rostral to caudal shift in electrode position produced a nasal to temporal change in the visual field (see Fig. 3).

These results show that the visual projection is well ordered along both the mediolateral and rostrocaudal axes of the tectum from the earliest stages of tectal innervation. In addition, retinal axons are arranged topographically through much of the developing pathway and seem to grow directly to their central target structures without projecting to ectopic locations along the way. The precision of fibre ordering must be better than $50\ \mu\text{m}$ in the tectum, as this is the entire mediolateral extent of the tectal neuropil at stage 39/40.

Our electrophysiological results differ from those of Gaze *et al.*¹⁰ in that (1) we obtained the first visual responses about 30 h earlier (stage 39 compared with stage 43/44) and (2) we found that these responses were topographically ordered when optic fibres first arrived in the tectum, instead of 2–3 days later (stage 40 compared with stage 46/47). The reason for the difference in the time of appearance of order is unclear. However, the brighter illumination and larger stimulus moved closer to the eye might account for the earlier responses in our

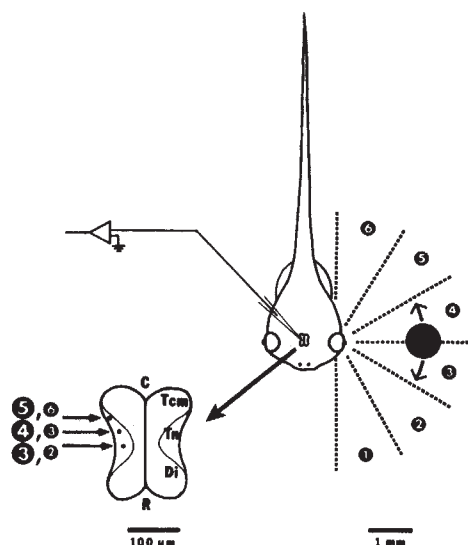


Fig. 3 Schematic representation of electrophysiological procedure used for mapping the visual field of young *Xenopus* tadpoles (stages 39–47). Animals were anaesthetized in 1:10,000 MS 222 (Sandoz) in 10% Holtfrete's solution. The skin was removed to expose the brain and the animal fixed on a 2-mm high Sylgard platform in a 4-cm plastic dish so that the left eye protruded slightly over the edge. 30° divisions were marked off around 180° on the bottom of the recording dish, illustrated by dashed lines, and the left eye was centred on the origin. A platinum-tipped electrode (5–10 μm) was placed in the neuropil region of the right tectum. The signal was fed into an amplifier and monitored over a loudspeaker. The stimulus, a 0.9-mm black ball (filled circle) attached to a thin (~0.1 mm) glass rod, was moved through 180°, from 1 to 6, at a distance of 2.5 mm from the eye and represented 20° visual arc. Both the animal and the stimulus were completely submerged. Responses were not due to water vibrations because they were eliminated in the dark. The intensity of the background illumination was $9.5 \times 10^4 \text{ erg cm}^{-2} \text{ s}^{-1}$. Responses were mostly multi-unit and, at early stage 40s, receptive field sizes were 30–90°, although smaller and larger values were sometimes encountered. Receptive fields >30° consistently had one 30° division which gave rise to a maximum response. For each electrode position, the 30° sector which elicited (1) the maximum response and (2) the next strongest were recorded. At later stages (45–47), the strength of responses increased, the background illumination required to elicit a response declined and receptive field sizes tended to be smaller. The diagram shows three electrode positions in the right tectum at stage 40 and the visual field sectors which elicited maximum (large numbers) and next strongest (small numbers) responses for each penetration. Tn, tectal neuropil; Tcm, tectal cell mass; Di, diencephalon; R, rostral; C, caudal.

study. (Visual responses could not be elicited in room light ($9 \times 10^2 \text{ erg cm}^{-2} \text{ s}^{-1}$) at early stage 40s and could not be driven by distant objects (over ~5 mm) until stage 45/46.)

These results show that the developing retinotectal map in amphibians differs from the regenerating one by demonstrating that diffuse projections, which commonly occur at the beginning of optic nerve regeneration^{13–16} are not made at the initial stages of development. Factors such as pathway ordering, sequential fibre outgrowth and target cell maturation probably contribute to the target-directed growth of developing fibres. In regeneration, when these processes have been disrupted, fibres might be forced to search out their targets by trial and error growth, thus giving rise to initially disordered maps. In the developing chick visual system where, in common with amphibian regeneration, a large number of optic fibres (4×10^6)²⁵ innervate a large (3–4 mm) post-mitotic tectum²⁶, a transient diffuse optic projection has recently been shown to exist²⁷. However, the projection was not examined until incubation day 10, 4 days after fibres have reached the tectum, which leaves the possibility that this aberrant projection might be absent initially.

We conclude that, like the formation of specific neuromuscular connections in the chick limb¹⁸, the topographic organiz-

ation of retinotectal projections arises by directed rather than trial and error nerve growth. The mechanisms responsible for this directed growth, whether due to fibre-fibre interactions, chemical gradients or spatio-temporal patterns of development, or other factors, have yet to be elucidated.

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Note added in proof: The segregation of dorsal and ventral retinal fibres observed here at early stages is consistent with the medial lateral tract segregation seen slightly later in development by Straznicki *et al.*²⁸.

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Absence of expression of OKT8 antigen on cultured human, calf and rat oligodendrocytes

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Monoclonal antibodies which recognize human suppressor T cells (OKT8) have been reported by Oger and co-workers to bind to cultured sheep oligodendrocytes¹. These authors speculated that an immune response directed at determinants shared by suppressor lymphocytes and oligodendrocytes could explain the decrease in both circulating blood suppressor T cells^{2,3} and oligodendrocytes⁴ in patients with multiple sclerosis. In view of the vital issue of potential cross-reactivity between oligodendrocytes and lymphocytes, we studied the binding of viable cultured calf⁵, rat⁶ and human⁷ oligodendrocytes using monoclonal antibodies to human T cells and monocytes⁸. We report here that we were unable to confirm the presence of shared determinants among oligodendrocytes and any leukocytes, including human T cells or monocytes. As the reported observations of lymphocyte-oligodendrocyte shared determinants could not be identified in three other species, including man, we found no evidence to support the hypothesis that such shared determinants are of importance in the pathogenesis of multiple sclerosis.

Mouse monoclonal antibodies which recognize human T lymphocytes (OKT3, OKT4, OKT6, OKT8) and monocytes and some null cells (OKM1) were obtained from Ortho Pharmaceuticals. Calf and rat oligodendrocytes were obtained by Percoll density gradient of trypsinized corpus callosum from young adult rats and calf, and were maintained in culture for 4–21 days^{5,6}. Human oligodendrocytes were obtained using a similar technique from human corpus callosum obtained at autopsy from the brains from two subjects (aged 9 months and 70 yr) without central nervous system (CNS) disease⁷. They were maintained in culture for 1–4 weeks. Cultures used for indirect immunofluorescence were carried on poly-L-lysine-coated coverslips and those used for binding by radioimmunoassay used poly-L-lysine-coated flat-bottomed microtitre plates, using 30,000 cells per well. Calf, rat or human oligodendrocytes were identified by their capacity to bind rabbit antigalactocerebroside (R-anti-GalC)^{5,9,10} or mouse (M) monoclonal antibody to GalC¹¹. We were unable to detect significant binding of any OKT reagents or OKM1 to cultured calf, rat or human oligodendrocytes in any of the experimental conditions used (Tables 1, 2). Significant binding to oligodendrocytes by positive control antisera (R-anti-GalC) and monoclonal M-anti-GalC antibodies were observed in the same experimental conditions⁴. In addition, the ¹²⁵I-labelled goat anti-mouse-Ig-F(ab')₂ (¹²⁵I-G-anti-M-Ig-F(ab')₂) detected binding of M-anti-GalC to GalC in solid-phase radioimmunoassay¹¹. These same OKT reagents bound to human blood¹² and thymic lymphocytes¹³. In the double-labelling experiments using OKT reagents and F(ab')₂ fragments of R-anti-GalC, GalC-positive oligodendrocytes never showed binding of the OKT reagents tested (Fig. 1).

We were unable to extend to cultured calf, rat or human oligodendrocytes the observation of Oger *et al.*¹ that mouse monoclonal antibodies to a subset of human T cells (OKT8) bind to a shared determinant on cultured sheep oligodendrocytes. We do not believe the failure to detect such binding in calf, rat or human oligodendrocytes is due to technical factors because we have: (1) used the same monoclonal antibodies at equivalent concentration (5–10 µg ml⁻¹); (2) simultaneously and successfully used these antibodies in the study of human blood¹² and thymic¹³ mononuclear cells; (3) shown that the second antibodies used successfully detected the binding of M-anti-GalC on oligodendrocytes (G-anti-M-Ig-FI and ¹²⁵I-G-anti-M-IgF(ab')₂). In addition, we tested cultures that were

Table 1 Binding of antibodies to oligodendrocytes tested by radioimmunoassay

	Rat (mean ± s.d.)	Calf (mean ± s.d.)
OKT3	284 ± 53	227 ± 26
OKT4	288 ± 40	238 ± 32
OKT8	274 ± 21	248 ± 35
OKM1	319 ± 37	214 ± 7
SP ₂	324 ± 48	244 ± 9
Normal mouse serum	323 ± 38	252 ± 23
M-anti-GalC	417 ± 51 (<i>P</i> < 0.05)	400 ± 40 (<i>P</i> < 0.01)
R-anti-GalC	1442 ± 167 (<i>P</i> < 0.001)	543 ± 93 (<i>P</i> < 0.001)
Normal rabbit serum	384 ± 14	216 ± 33

Rat or calf oligodendrocytes (4–8 days *in vitro*) cultures were incubated for 30–60 min with heat-inactivated (56 °C for 30 min) normal rat or calf serum (1:10 dilution) to block any potential sites of interaction with the Fc portion of immunoglobulin. After flicking serum and medium, cultures were incubated at 23 or 4 °C for 90 min with mouse monoclonal antibodies (5–10 µg ml⁻¹), washed and then incubated with ¹²⁵I-labelled goat anti-mouse immunoglobulin F(ab')₂ (G-anti-M-IgF(ab')₂; NEN) for 90 min. The cells were washed again and collected by lysing them with 1 M NaOH. Radioactivity was determined in an autogamma counter (Packard). Positive and negative controls consisted of cultures incubated with: (1) mouse monoclonal anti-GalC, supernatants of a non-producing SP₂ mouse myeloma¹¹ and normal mouse serum (dilution 1:500) followed by ¹²⁵I-G-anti-M-IgF(ab')₂; (2) R-anti-GalC (dilution 1:50) and normal rabbit serum (dilution 1:50) followed by ¹²⁵I-labelled staphylococcal protein A (NEN). All individual studies were performed at least in triplicate and binding of each monoclonal antibody was tested on three or more occasions. The data show mean ± s.d. of quadruplicate c.p.m. from a representative experiment. A *t*-test was performed between each experimental group and normal serum control.

highly enriched for oligodendrocytes (R-anti-GalC⁺ cells) and tested cultures at various times *in vitro* to allow for regeneration of trypsin-sensitive membrane components. We also tested the binding at 23 and 4 °C (to inhibit any potential of capping, shedding and/or endocytosis) and blocked any nonspecific Fc-dependent binding of mouse immunoglobulin which might obscure additional specific binding by any of the monoclonal antibodies.

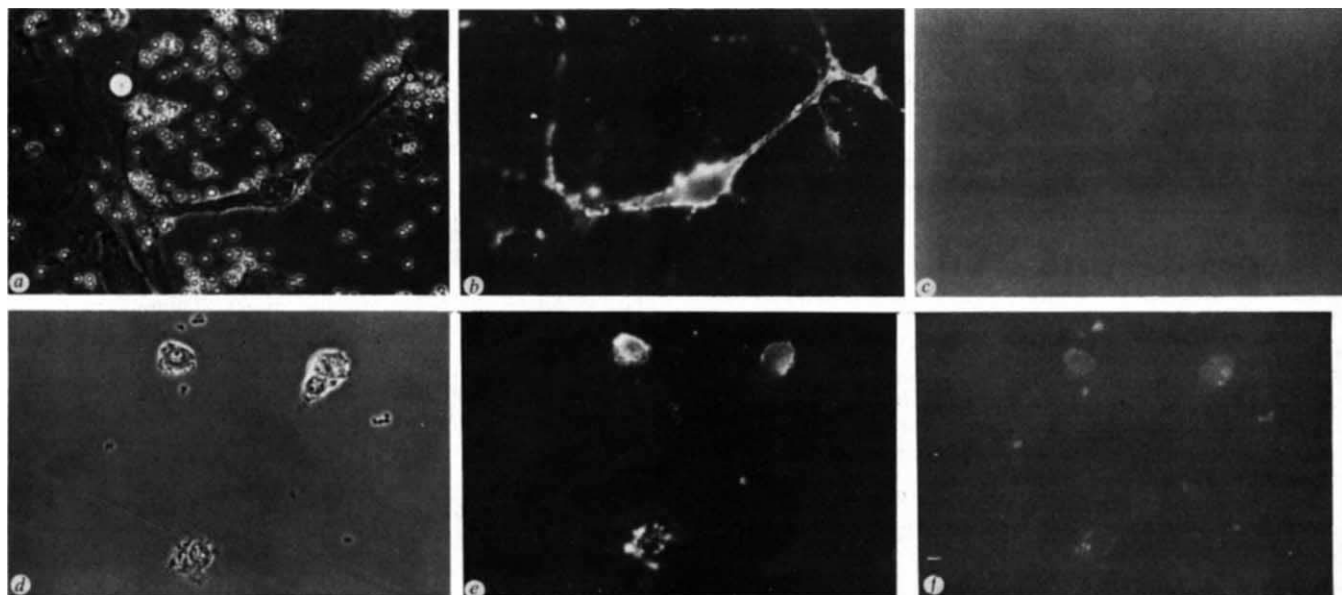


Fig. 1 Rat oligodendrocytes in culture (7 days *in vitro*) viewed by: a, phase; b, rhodamine (GalC); c, fluorescein (OKT8) microscopy, and human oligodendrocytes in culture (8 days *in vitro*, from 70-yr-old human autopsy specimen) viewed with: d, phase; e, rhodamine (GalC); f, fluorescein (OKT8). GalC⁺ cells (b, e) were OKT8- (c, f) and latex- (a).

Table 2 Binding of antibodies to oligodendrocytes tested by indirect immunofluorescence

Antibody	Specificity to human mononuclear cells	Rat	Calf	Human
OKT3	Pan blood T	—	—	—
OKT4	Helper T	—	—	—
OKT6	Common thymocyte	—	—	ND
OKT8	Suppressor T	—	—	—
OKM1	Monocytes and K cells	—	—	—
R-anti-GalC		+++	+++	+++
M-anti-GalC		++	+	+

The coverslips of rat, calf and human oligodendrocytes were incubated in heat-inactivated normal rat, calf or human serum (1:10 dilution) for 30 min to block any potential sites of interaction with Fc portion of immunoglobulin. After flicking the serum and medium, they were incubated with mouse monoclonal antibodies at 23 or 4 °C for 30 min, washed and then incubated with fluorescein-conjugated goat anti-mouse immunoglobulin (1:50 dilution, G-anti-M-Ig-Fl, Tago) for 30 min. After washing, the cells were post-fixed with acid alcohol for 20 min at -20 °C, washed and the coverslips mounted with glycerol-phosphate-buffered saline^{5,6,9,10}. They were examined with phase and fluorescence optics (Zeiss). Parallel cultures were routinely examined for capacity to bind R-anti-GalC and/or M-anti-GalC. The latter also provided a positive control for the G-anti-M-Ig-Fl second antibody. In some cultures, we performed double-labelling experiments in which the cultures were simultaneously incubated with OKT reagents and F(ab')₂ fragments of R-anti-GalC (prepared by Dr Alan Schreiber) followed by G-anti-M-Ig-Fl and rhodamine-conjugated F(ab')₂ fragments of goat anti-rabbit immunoglobulin F(ab')₂ (F(ab')₂-anti-R-IgF(ab')₂, 1:100 dilution, Cappel). In some experiments, cells were first incubated with latex particles (1.1 µM, Dow) to identify macrophage-microglia¹⁹. Negative controls included SP₂ supernatant, normal mouse serum followed by second antibody. —, Negative; +, weakly positive; ++, moderately positive; +++, strongly positive binding by immunofluorescence; ND, not done.

There are several possible differences to consider between our study and that of Oger *et al.*¹. These investigators used cells obtained by trypsinization and sucrose gradient centrifugation¹⁴, whereas we used cells obtained by trypsinization and Percoll gradient centrifugation⁵⁻⁷. It is conceivable but unlikely that this difference is important, as both methods result in healthy viable cultures with cells that express surface GalC and cytoplasmic myelin basic protein^{5-7,14}. A second difference might be differences in culture media, but again we consider this unlikely because our procedures result in healthy, metabolically active cells^{5-7,15,16}. Species difference (sheep as opposed to calf, rat and human) could be important although one would expect calf, rat and certainly human oligodendrocytes to share the OKT8 determinant with human suppressor T cells if sheep oligodendrocytes share this determinant. In addition, the pathogenic significance in human disease of an antigenic determinant shared by human lymphocytes and sheep oligodendrocytes but not human oligodendrocytes is questionable.

In addition, there was the recent, very brief account of negative OKT8 immunostaining of oligodendrocytes in frozen sections of human white matter, optic nerve, multiple sclerosis plaques and oligodendroglioma¹⁷. In view of the problems inherent in identifying surface constituents of cells in frozen sections, especially in brain (that is, GalC was identified in myelin in sections, but was not detected on the surface of oligodendrocytes¹⁸, whereas by using cultures of viable CNS cells in monolayers, oligodendrocyte surfaces are clearly GalC⁺)¹⁹, our culture system is more closely comparable with the experiments by Oger *et al.*¹.

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A new source of embryonic lymphocytes in the mouse

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The source of stem cells within the early mammalian embryo has not been identified. The yolk sac, once thought to provide the early stem cells in birds, is now seen more as a primitive effector organ capable of allorecognition, natural cytotoxic reactions and elaboration of cytokines¹⁻⁴. In lower vertebrates the source appears to be within a region delineated by the anterior limbs, foregut and mesonephros⁵⁻⁹. That region defines the boundaries of the developing omentum, a fold in the peritoneum. As the omentum is known to develop lymphoid cells postnatally^{10,11} we have now examined the omental rudiment for the presence of lymphoid cell precursors. Our experiments provide evidence that the presumptive omentum of the 13-day mouse embryo is capable of histotypic differentiation into a reticular organ containing a significant number of Thy 1⁺ lymphocytes, which suggests that the omentum may represent a new primary lymphoid organ in the mouse.

The presumptive omentum first becomes distinct from the splenic and pancreatic rudiments in mouse embryos at 13 days (equivalent, approximately, to 5-day chick or 6-week human embryos). By dissection of 13-day embryos we were thus able to isolate the loose mesothelial sheet representing the omental primordium. For comparison we also isolated splenic and thymic rudiments from these same embryos. The capacity of the isolated structures for lymphoid differentiation was assessed by transplantation into the anterior eye chamber of histocompatible, syngeneic (BALB/c → BALB/c) irradiated adult mice. We had previously shown that in these conditions self-differentiation of grafted embryonic rudiments can take place, but that the irradiation of the host animals prevents migration of host lymphoid cells into the grafts. Thymus rudiments developed within 7 days into typical mature thymus grafts filled with lymphocytes¹² while, as expected¹³, grafts of the 13-day embryonic spleen grew well, showed erythroid differentiation, but failed to contain lymphocytes. Transplants of the omental anlage, examined 7 days after transplantation, developed into well-vascularized reticular organs filled with lymphoid cells and showing erythropoiesis. Occasionally, pancreatic acini were seen at the periphery of the graft, suggesting incomplete separation of presumptive pancreas. A total of 16 omental grafts were examined by histology: each had become lymphoid. All thymus grafts examined in this series ($n = 4$) were lymphoid; in contrast, no splenic graft ($n = 7$) showed visible lymphocytes on histological examination. Representative sections of grafted embryonic thymus, spleen and omentum are shown in Fig. 1.

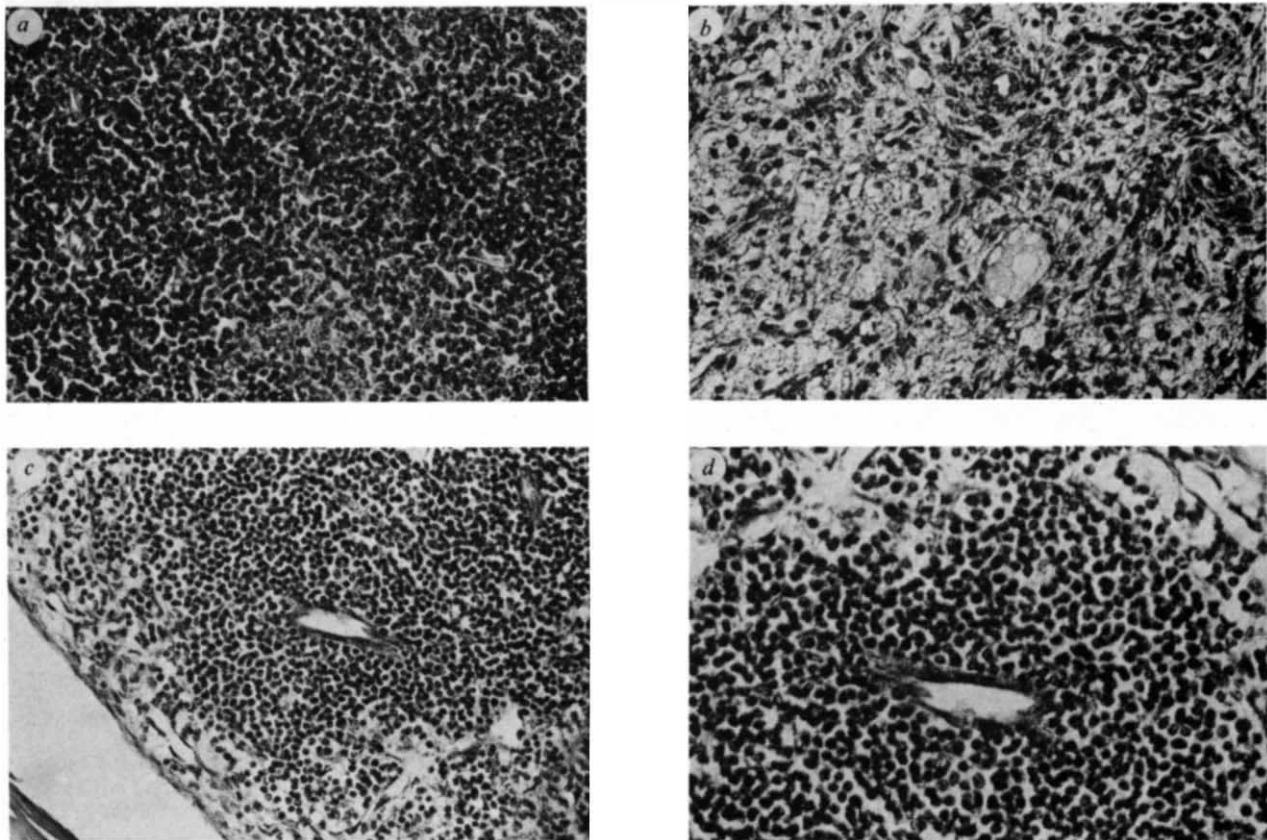


Fig. 1 Histological preparations stained with haematoxylin and eosin. Thymic (a), splenic (b) or omental (c, d) rudiments were dissected from 13-day mouse embryos and transplants were made into the anterior eye chamber of adult mice irradiated with X rays (750R). Grafts were fixed after 7 days. Note that virtually the entire thymus graft consists of small, darkly stained lymphocytes, while the spleen lacks these cells. The omental rudiment contains a dense concentration of lymphocytes centrally, with reticular/haematopoietic tissue restricted to the periphery of the section. Omental lymphocytes are slightly larger than thymic lymphocytes as seen both in these photomicrographs and by flow cytometric analysis. a-c, $\times 220$; d, $\times 350$.

Previous experiments¹³ suggested that the observed omentum-associated lymphocytes (O-cells) were probably derived from the grafted omental tissue. To verify this we examined cells isolated from grafts of BALB/c rudiments grown in (BALB/c $\times$ CBA)F₁ hosts for expression of *H-2* haplotypes. Graft-derived cells would be expected to express only *H-2^d* while host-derived cells should express both *H-2^d* and *H-2^k* antigens. O-cells obtained from 7-day grafts, examined by flow cytometry, were found to express only the *H-2^d* haplotype.

To determine the type of lymphocyte formed in the omental grafts, cell suspensions prepared from transplanted rudiments were labelled with either a fluoresceinated anti-Thy 1.2 serum or with fluoresceinated rabbit anti-mouse immunoglobulin serum (we used both a rabbit anti-IgG heavy plus light chain serum and a rabbit anti-IgM μ -chain-specific reagent). Cells obtained from 7-day thymus and spleen grafts were tested in a similar manner. As expected, isolated thymus cells showed $>90\%$ labelling with the anti-Thy 1 reagent (see also refs 14–16) and no ($<5\%$) labelling with the anti-immunoglobulin sera. Spleen grafts yielded only a few cells which failed to label with either reagent. Cells obtained from grafts of the omental rudiment showed 50–90% binding with the anti-Thy 1 serum and no specific labelling with the anti-immunoglobulin reagents (Fig. 2).

Additional studies were carried out using monoclonal antibodies against Lyt antigens (Becton-Dickinson anti-Lyt 1 and anti-Lyt 2). Lymphocytes obtained from omental rudiment were clearly Lyt-2 negative. A weak but consistent labelling with the anti-Lyt 1 reagent was observed, but the overlap of labelled with unlabelled cells prevents calculation of a meaningful labelling percentage (see also ref. 17). Further evaluation with alloantisera and different monoclonal reagents is indicated.

Two experiments were carried out in which BALB/c omental or thymic rudiments were transplanted into irradiated AKR mice. Although this combination involves allografting, immunological rejection in the anterior eye chamber is sufficiently delayed to permit normal development of the grafts for 7 days. Cells obtained from the transplants were tested both for Thy-1 and *H-2* antigens. Cells from the graft would be expected to express Thy 1.2 and *H-2^d* antigens, while donor cells would express Thy 1.1 and *H-2^k* determinants. Flow cytometric analysis showed that the cells from both thymic and omental grafts expressed *H-2^d* and Thy 1.2 but failed to be labelled by either anti-*H-2^k* or anti-Thy 1.1 sera. Labelling with the anti-Thy 1 serum was somewhat weaker for omentum-derived lymphocytes than for thymus-derived lymphocytes as assessed by either total fluorescence per cell or by the ratio of fluorescence to low angle light scatter signals.

The search for the origin of lymphoid stem cells in the developing vertebrate embryo has given rise to much confusion (refs 1, 4; see ref. 15 for discussion). There is no *a priori* reason to assume that there is only a single origin of lymphoid cells, given the many subsets of lymphocytes such as B and T cells (and perhaps O-cells), the multiple effector cell activities and regulatory functions, and the changing pattern of defence mechanisms during phylogeny and ontogeny. As yet there is no view of lymphoid embryogenesis which will readily incorporate the present results.

Many questions are raised by our observations: (1) Are stem cells present in the omental region at earlier times in development? Stem cell migration into the thymus, for example, occurs on days 11–13, perhaps the pre-omental tissue (or anterior splanchnic mesodermal plate) is the site from which stem cells migrate to the developing thymus rudiment. (2) How much of

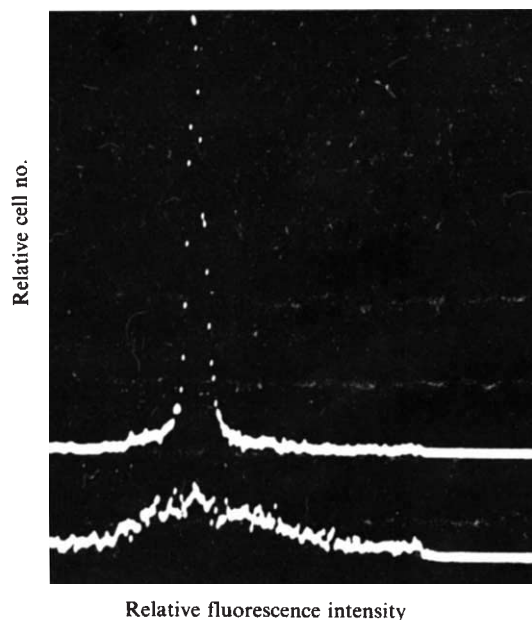


Fig. 2 Flow cytometric analysis of cells obtained from BALB/c omental or thymic rudiments 7 days after transplantation into the anterior eye chamber of adult, irradiated AKR mice. Cells were released from the grafted tissue by teasing, leaving behind the reticular and tissue-adherent cells. Cells were exposed sequentially to monoclonal anti-Thy 1.2 (IgM; NEN) and rabbit anti-mouse IgM (μ -chain specific Cappel, fluorescein isothiocyanate-labelled) sera. Flow cytometric studies were carried out on a Becton-Dickinson FACS-IV equipped with a 5-W argon ion laser tuned to 488 nm and operating at 100 mW output. Cells were passed through a 70 μ m orifice, assessed for low angle light scatter (data not shown) and intensity of fluorescence emission at 515 nm.

the region we have designated as pre-omental contributes directly to later splenic development (days 14–16), and would that region represent the only part of the omental anlage that contains lymphoid stem cells? (3) Is the differentiation of Thy 1⁺ cells completely independent of thymic influence or are there thymic humoral factors that are essential for this differentiation to occur? (4) Is the amount of Thy-1 antigen expressed on omentum-derived lymphocytes high enough to exclude the possibility that Thy-1 is not a marker of stem cells in general (including 0-cells) rather than only of T-cell subsets^{14,16}? (5) Perhaps specific functional activities are carried out by the particular lymphocytes developed from the omental anlage, in which case, do they possess specific properties generally considered characteristic of B cells, T cells or null (natural cytotoxic) cells? (7) Is it possible that the omental lymphoid system has unique properties of its own, seen by expression of specific differentiation markers, by selective response to antigens, by unique patterns of mitogen sensitivity or by novel immunological surveillance reactions?

Our experiments simply demonstrate that at day 13 of mouse development lymphoid cell precursors capable of differentiating into Thy 1⁺ cells exist in the omental mesenchyme; they do not yet address their prior origin, their developmental fate or their functional capacity. They focus attention on a new site of localization of murine lymphoid stem cells whose role in the structural and functional differentiation of the mammalian immune system must now be further investigated.

We thank Professor Kazimierz Dux for stimulating discussions which led directly to the initiation of this research; and we acknowledge L. W. Morrissey for performing the flow cytometric analysis. This work was supported by NIH grants EY 3243 and AI 14607.

Note added in proof: The Lyt-1 monoclonal antiserum used in our study has now been reported to mark not only T cells but also at least one subset of B cells¹⁸.

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X rays may be twice as potent as γ rays for malignant transformation at low doses

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The introduction in the 1950s of ⁶⁰Co teletherapy units and megavoltage X-ray accelerators for radiotherapy prompted several studies which showed that the relative biological effectiveness (RBE) of orthovoltage X rays, compared with ⁶⁰Co γ rays, was ~1.1–1.2 (refs 1, 2). Subsequently, radiation therapists confirmed that the effect of established treatment protocols using orthovoltage X rays could be duplicated with the higher energy radiation by increasing the dose by ~10%, when radiation doses are of the order of tens of grays (or thousands of rads). The dependence of biological effectiveness on photon energy led to recommendations in which the quality factor, *Q*, which is an RBE for radiation protection purposes, was set at unity for X and γ rays as well as for electrons or other directly ionizing particles having a linear energy transfer (LET) of <3.5 keV μ m⁻¹. Over the past decade, however, several studies have shown differences in RBE between various low-LET radiations having LET values within the range designated for standard radiation^{3–6}. Underbrink *et al.*⁷ found an RBE for orthovoltage X rays relative to ⁶⁰Co γ rays of ~2 at 0.04 Gy, for induction of pink mutations in the stamen hairs of *Tradescantia*. Schmidt *et al.*⁸ scored chromosome aberrations in human lymphocytes, and at 0.25 Gy found the RBE of 200-kVp X rays relative to 3-MeV electrons to be also ~2. Here we have compared ⁶⁰Co γ rays and orthovoltage X rays over the dose range 0.03–1.5 Gy using malignant transformation in mammalian cells *in vitro*^{9–13} as an end point. Our findings indicate that whereas the transformation incidence seems similar for X and γ rays at high doses, the malignant potential of X rays is about twice that of γ rays at 0.03 Gy.

In vitro assay systems using mammalian cells are useful for assessing the potential for malignant transformation of radiation of various energies and in establishing the RBE for the purpose of radiation protection, as carcinogenesis is the late effect of principal concern. The hamster embryo clonal assay system allows the identification, at low incidence, of cells that have undergone a malignant transformation via the altered morphology of the clone produced. We have used this assay system to compare the malignant potential of orthovoltage X rays with

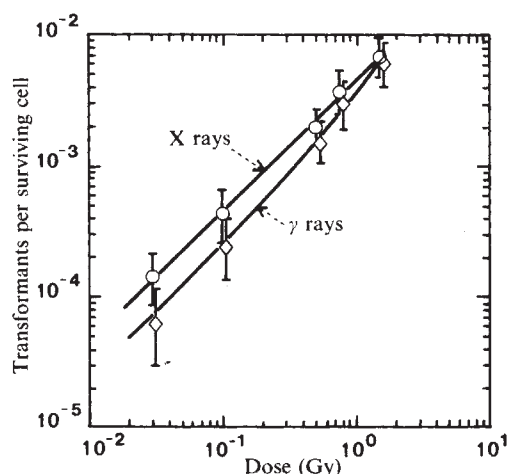


Fig. 1 Transformants per surviving cell as a function of dose for hamster embryo cells exposed to graded doses of X or γ rays. Data were pooled from several experiments. The error bars represent 95% confidence limits calculated assuming a Poisson distribution of the number of transformants (Fig. 3 legend). The curves represent linear-quadratic dose-effect relationships fitted to the data (see text). Primary cultures were established by progression dissociation of fresh minced tissue⁹⁻¹² and the cells were cultured and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. Details of the methodology have been published previously⁹⁻¹². Briefly, cells were seeded into tissue culture flasks at a density of 10^3 cells per 60 mm Petri dish and allowed to attach for 24 h before being irradiated. The source of γ rays was a ^{60}Co teletherapy unit, while the X-ray source was a 300-kVp Siemens Stabilipan machine operating at 12 mA, with added filtration of 0.2 mm Cu. Doses were calculated from measurements made with a Victoreen R-meter, the calibration of which could be traced to the National Bureau of Standards through the secondary standard at the Memorial Sloan-Kettering Cancer Center. The dose rate for X and γ rays was 0.15 and 0.33 Gy min⁻¹, respectively.

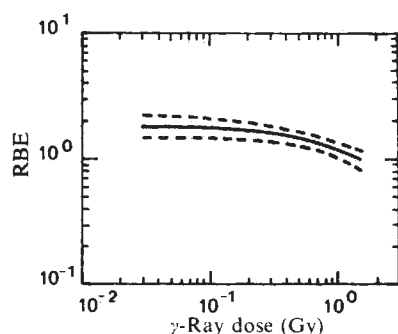


Fig. 2 The relative biological effectiveness (RBE) of orthovoltage X rays relative to ^{60}Co γ rays, calculated from the linear-quadratic relationships of Fig. 1. The broken curves indicate a confidence interval of 1 s.d. for the estimated RBE.

that of ^{60}Co γ rays. Explants of mid-term golden hamster embryos were used for these experiments.

A number of large, self-contained experiments were carried out and in each experiment X and γ rays were compared at a given dose or series of doses. The number of doses that could be compared in a given experiment was limited by the large number of flasks that had to be exposed in order to obtain a sufficient number of transformed clones. For logistical reasons, 1,000 dishes for each type of radiation was the practical limit.

After the completion of all irradiations, the flasks were incubated at 37 °C for 10 days before being fixed and stained with Giemsa. The number of normal and transformed clones per flask was counted; dishes were coded and randomized to eliminate observer bias. Transformed clones were identified by their changed morphology, that is, densely-stained piled-up cells,

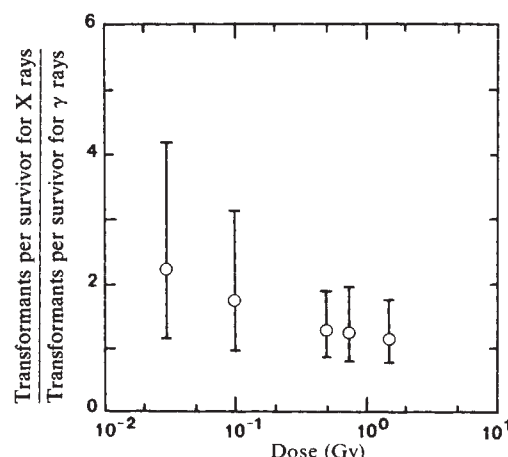


Fig. 3 Ratio of the number of transformants per surviving cells produced by X rays to the number produced by γ rays as a function of dose. The vertical bars represent 95% confidence intervals, and were calculated by assuming that the number of transformants in an experiment is a random variable with a Poisson probability distribution, as follows. Statistical treatment: For this figure and Fig. 1, assuming the number of transformants (x) observed in an experiment is a random variable with a Poisson probability distribution:

$$p(x|\lambda) = e^{-\lambda} \lambda^x / x! \quad (1)$$

where the unknown λ is the expected number of transformants, the basic statistical problem is to estimate λ (point and/or interval estimation) given an observation x . It can be shown¹⁵ that under the most conservative assumption (no previous information on λ) the conditional probability distribution of λ , given x , is:

$$g(\lambda|x) d\lambda = e^{-\lambda} \lambda^x / x! d\lambda \quad (2)$$

One can then estimate λ , for example by the value λ_0 which will maximize $g(\lambda|x)$ (here this will be identical to the maximum likelihood estimator). The calculations for this figure relate to a slightly different problem; given two observations, x_2 and x_1 , we need to estimate confidence intervals for the ratio $r = \lambda_2/\lambda_1$ of the expected frequencies associated with x_2 and x_1 , respectively. From the result of equation (2), it is easy to show that the distribution of r is given by:

$$p(r) dr = \frac{\Gamma(x_1 + x_2 + 2)}{\Gamma(x_1 + 1)\Gamma(x_2 + 1)} \frac{r^{x_2}}{(1+r)^{x_1+x_2+2}} dr \quad (3)$$

and furthermore that:

$$\int_0^{r_\alpha} p(r) dr = F_{m,n} \left(\frac{n}{m} r_\alpha \right) \quad (4)$$

where $F_{m,n}$ is the F -distribution with m and n degrees of freedom given by $m = 2(x_2 + 1)$ and $n = 2(x_1 + 1)$. Equation (4) was used to find, at a given confidence level, α , r_α and r'_α for which:

$$\int_{r'_\alpha}^{r_\alpha} p(r) dr = \alpha \quad (5)$$

The intervals $[r'_\alpha, r_\alpha]$ shown in this figure are further normalized per surviving cell, respectively (see Table 1).

showing a loss of contact inhibition and a random orientation, particularly at the border of the clone⁹⁻¹². The correlation between this morphology of the transformed cells and their malignancy has been documented elsewhere⁹⁻¹². Transformation incidence was scored as the number of transformants per surviving cell¹⁰⁻¹².

Figure 1 gives the data from all the experiments; data from replicate experiments at the same dose were pooled. The curves represent linear-quadratic dose-effect relationships fitted to the weighted data by the least-squares method. Using standard statistical procedures, the X-ray data were found to be consistent with transformation incidence being only a linear function of dose. The best-fit curves were $(\pm 1 \text{ s.d.}): E_X = (4.5 \pm 0.4) 10^{-3} D_X$ and $E_\gamma = (2.4 \pm 0.5) 10^{-3} D_\gamma + (1.3 \pm 0.7) 10^{-3} D_\gamma^2$ where E_X and E_γ are the number of transformants per surviving cell

Table 1 Transformation of hamster embryo cells *in vitro* by X and γ rays

X rays					γ rays			
Dose (Gy)	No. of clones	SF (PE)	No. of transformed clones	Transformed clones/surviving cells	No. of clones	SF (PE)	No. of transformed clones	Transformed clones/surviving cells
0	17,155	(3.2)	0	0	17,950	(3.5)	0	0
1.5	5,010	0.73	35	6.99×10^{-3}	4,999	0.78	30	6.0×10^{-3}
0.75	8,500	0.84	32	3.76×10^{-3}	8,401	0.88	25	2.98×10^{-3}
0.5	20,880	0.90	42	2.01×10^{-3}	21,891	0.97	34	1.55×10^{-3}
0.1	41,802	0.92	18	4.31×10^{-4}	48,875	0.98	12	2.45×10^{-4}
0.03	132,600	0.98	19	1.43×10^{-4}	140,200	0.98	9	6.42×10^{-5}

SF, surviving fraction = no. of clones counted/(no. of cells plated $\times$ plating efficiency) (PE). PE = (no. of clones counted/no. of cells plated) $\times$ 100.

resulting from doses D_x and D_γ (in Gy) of X and γ rays, respectively.

Figure 2 shows the RBE of orthovoltage X rays relative to ^{60}Co γ rays as a function of dose calculated from the curves of Fig. 1; it also shows the confidence limits for the RBE corresponding to 1 s.d. and calculated by propagating the errors (variance, covariance) in the estimated parameters. Figure 2 shows that the RBE for orthovoltage X rays is ~ 1.1 for high doses, but increases to ~ 1.9 at the low end of the observed dose scale.

Figure 3 shows an alternative way of presenting the data which is based on a non-parametric analysis and therefore does not invoke any particular form for the dose-response relationship. It shows at a given dose the ratio of the number of transformants per surviving cells for X rays relative to γ rays; the 95% confidence limits shown in Figs 1 and 3 were estimated under the usual assumption that the number of transformants at a given dose has a random Poisson distribution (see Fig. 3 legend). Within the statistical limitations of the present experiments the assumption of a constant ratio, independent of dose, cannot be rejected; however, the trend of the individual ratios indicates an increase to a value of 2 at the lowest dose.

The radiobiological studies reported here demonstrate that a significant difference in effectiveness per dosage unit exists at low doses for two different radiations that fall within the low-LET range 0.2–3.5 keV μm^{-1} (that is, the so-called standard radiation when calculating RBE and Q values). These studies also confirm previously published results^{7,8} obtained for chromosomal aberrations or mutation in plant cells. In view of the diversity of cell types and end points used, it is reasonable to assume that the above conclusions apply more generally. These observations also agree with theoretical predictions based on microdosimetric considerations. Bond *et al.*¹⁴ calculated the low-dose limiting value of the RBE for X rays relative to γ rays to be ~ 2 . This calculation was based on microdosimetric data for energy absorption in volumes comparable with the dimensions of the cell nucleus. Our data confirm that the spectrum of energy depositions has important biological implications at low doses.

In the context of radiation protection, these studies suggest the need to define the reference radiation more sharply than at present in order to avoid unnecessary ambiguity, at least at low doses. It has been often suggested that it would be better to choose for the reference radiation one of high LET for which the variation of biological effect with dose rate is minimal. The difficulty in this approach would be the availability of a suitable source for every laboratory. Therefore, it may be still more practical to retain ^{60}Co γ rays as the reference as, in addition to being widely available, it approximates the theoretical lower limit of biological effectiveness. Finally, while it is customary to present data in terms of RBE, an alternative analysis such as that of Fig. 3 may be useful, as the effect of equal doses of different radiations can be readily assessed.

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Phenotypic and functional heterogeneity of human cloned natural killer cell lines

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Extensive efforts have recently been made to characterize cells capable of mediating natural killing activity (see ref. 1 for review) and increasing evidence has arisen that these cells were heterogeneous^{2,3}. By using the methods we have recently developed for cloning natural killer (NK) cells derived from peripheral blood⁴, we have analysed the heterogeneity of human NK cells. Seven cell lines showing NK activity were established and studied for 4 months. Their phenotype was determined with a series of monoclonal antibodies; anti-T1, -T3, -T4, -T8, -T11, -T12, Mo1 and each cell line appeared to have a unique phenotype. Moreover, whereas some of these lines could only kill K562 cells, the standard assay of NK activity, others displayed a broad but distinct spectrum of reactivity against a variety of human tumour and viral transformed cell lines. Taken together, these results demonstrate the phenotypic and functional heterogeneity of NK effector cells which has recently been suggested in both human and murine systems^{2,3,5}.

In human peripheral blood, most NK cells are found within a small population of large granular lymphocytes (LGL) which are distinct from mature T cells, B cells and monocytes^{1,6}.

However, it has been suggested that activated T cells could mediate natural killer activity^{7,8}. In order to obtain a broad variety of cell lines with NK activity, we carried out the following series of experiments. Peripheral blood mononuclear cells (PBMC) were obtained from a volunteer donor by Ficoll-Hypaque density gradient centrifugation and divided into three groups. One group of 200 cells was cloned immediately by limiting dilution at one cell per well on a feeder layer of autologous irradiated PBMC and stimulated by phytohaemagglutinin (PHA). The second and third groups of 2×10^6 PBMC were stimulated by either 2×10^6 autologous (Laz 461 cell line) or allogeneic (Laz 388 cell line) irradiated Epstein-Barr virus (EBV) transformed B cells. Seven days later, the latter two groups were restimulated by their respective B-cell lines and on day 11, 200 cells from each group were cloned at one cell per well on a feeder layer of autologous irradiated PBMC plus either irradiated Laz 461 or Laz 388 cells. Additional lines were developed using purified LGL as the starting population which were also divided into three groups and stimulated by either PHA, Laz 461 or Laz 388. They were not preimmunized in the autologous and allogeneic system and consequently, all LGL were cloned on day 0. The feeder layers were identical to that described above for PBMC. Therefore, a total of 600 PBMC and 600 LGL were cloned using three distinct types of stimulation. Following initial cloning, all cultures were fed every 3 days with medium containing lymphocyte conditioned medium (15% final dilution). Colonies appeared after 3 weeks and were first screened on the basis of their ability to kill K562 cells. The effector/target ratio used was 20:1 and only colonies displaying high NK activity ($\geq 60\%$) were expanded for further characterization. The cloning efficiency was $\sim 5\%$ for the three groups of LGL but all the colonies tested had NK activity. With PBMC, the cloning efficiency was higher ($\sim 40\%$ in all three groups) and only the most rapidly growing colonies were tested for NK activity against K562. As expected from previous observations, variable percentages of clones derived from PBMC displayed NK activity. The percentage of NK active clones varied depending on the type of stimulation used: 14% (3 out of 21) following PHA stimulation; 6% (3 out of 46) following allogeneic stimulation with Laz 388; 40% (12 out of

30) following autologous stimulation with Laz 461. In these two latter systems, none of the NK colonies were able to kill immunizing cells Laz 388 or Laz 461.

Following this screening for NK activity, seven selected colonies maintained rapid proliferation and were expanded for subsequent phenotypic and functional studies. They were termed JT3 to JT9 and the origin of these cell lines is presented in Table 1 in addition to JT1 which was generated in previous experiments. As shown, the JT lines are derived from different cell fractions following various proliferative stimuli. JT1 has now been continuously growing in culture with stable phenotype and function for 8 months and JT3 to JT9 for 4 months. All these human lines are strictly dependent on lymphocyte conditioned medium for proliferation, as has previously been reported for murine NK clones^{9,10}.

The phenotype of the eight cell lines was determined with a series of monoclonal antibodies which have previously been characterized (Fig. 1). The reactivity of all antibodies except for anti-Mo1 has been stable in at least three determinations over a 5-week period of time. The expression of Mo1 appeared variable from one determination to another, although in most cases, low amounts of antigen were present at the cell surface. The cell lines JT1, 3, 4, 5 which were derived from a T3 negative population present in peripheral blood do not express the T3 antigen. However, these lines have distinct phenotypes: JT1 does not express any T-cell-related antigens; JT5 only reacts with anti-T11; JT3 reacts with anti-T11, anti-T1, and weakly with anti-T12; JT4 is similar to JT3 but does not express T1. JT6, 7, 9 which were derived from PBMC express the T3 antigen. JT7 and JT9 have the phenotypic characteristics of common mature T lymphocytes present in peripheral blood. JT7 expresses T3 and T4 like helper cells¹² whereas JT9 expresses T3 and T8 like cytotoxic/suppressor cells¹⁶. JT6 also expresses T3 and T8 but does not react with anti-T1 or anti-T12. JT8, derived from PBMC, does not express T3 but reacts with both anti-T8 and anti-T11. In addition, subclones of JT1, JT3 and JT9 were tested for expression of the HNK-1 antigen which is present on a majority of peripheral blood large granular lymphocytes¹³. HNK-1 was found to be present only on JT9 which further underlines the heterogeneity of human NK

Table 1 Generation of cloned cell lines and their natural killer-like activity

	Origin	Initial stimulation	K562	Molt-4	HL60	U937	Laz 221	Laz 388
JT1	NC	PHA	+++	+++	+++	+++	+	-
JT3	NC	Laz 461	+++	++	+	+	+	-
JT4	NC	PHA	++	-	-	-	-	-
JT5	NC	Laz 388	+++	+	++	++	-	-
JT6	PBMC	Laz 388	++	-	-	-	-	-
JT7	PBMC	PHA	+++	-	-	-	-	-
JT8	PBMC	Laz 461	+++	+	++	++	-	+
JT9	PBMC	PHA	++	+++	+	+++	+++	+

PBMC were obtained from a volunteer donor by Ficoll-Hypaque density gradient centrifugation. LGL were obtained by complement lysis ($\times 2$) with anti-T3 (specific for mature T cells), anti-B1 (specific for B lymphocytes) and anti-Mo2 (specific for macrophages) monoclonal antibodies as previously described⁴. All cultures were done in RPMI 1640 containing 20% human pooled AB serum. PBMC and LGL were cloned by limiting dilution at one cell per well on a feeder layer of 25,000 irradiated (5,000 rad) PBMC. PHA was used on day 0 at a final concentration of $2 \mu\text{g ml}^{-1}$. When autologous EBV transformed B cells (Laz 461 cell line) or allogeneic EBV transformed B cells (Laz 388 cell line) were used in the cloning procedures, 15,000 irradiated (5,000 rad) cells were added in each well. Following cloning all cultures were fed every 3 days with culture medium containing 15% LCM (final dilution). Preparation of LCM has been reported in detail previously⁴. Briefly, PBMC were stimulated with a combination of PHA ($5 \mu\text{g ml}^{-1}$), PMA (5 ng ml^{-1}) and irradiated B-cell lines. Cells were washed three times after 4 h to remove PMA and PHA excesses and resuspended in fresh medium. After 36 h, the culture supernatants were collected, filtered and stored at -70°C . The cytotoxicity of the various cell lines was determined using a chromium release assay which has been described previously⁴. Briefly, 2,500–5,000 ^{51}Cr -labelled target cells were incubated with various numbers of effector cells at 37°C in V-bottom microtitre plates. The plates were spun and cytotoxicity measured by the release of ^{51}Cr into the supernatant after a 4-h incubation. Maximal release (MR) was determined by addition of 1% NP40 detergent. Spontaneous release (CR) never exceeded 20%. Specific cytotoxicity was calculated as follows:

$$\% \text{ Specific lysis} = \frac{\text{Exp} - \text{SR}}{\text{MR} - \text{SR}} \times 100$$

The specific cytotoxicity is expressed as follows: -, $\leq 5\%$; +, 5–30%; ++, 30–60%; +++, $\geq 60\%$. Cytotoxicity assays were performed in RPMI containing 10% human pooled AB serum, effector cells were washed twice and resuspended in this medium before the experiments. The effector/target ratio is 12:1 in all experiments. K562 was established from a patient with chronic myelogenous leukaemia. Molt-4 is a T-cell leukaemia cell line. U937 is a histiocytic cell line. Laz 221 and Laz 388 were derived from the same patient. Laz 221 is a CALLA-positive ALL line whereas Laz 388 is an EBV transformed B-cell line.

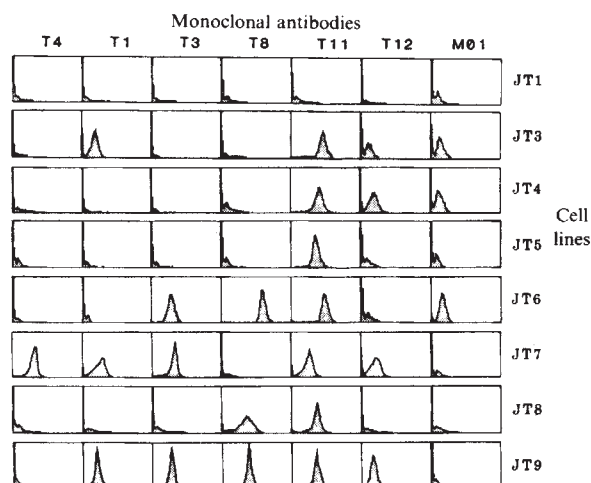


Fig. 1 Phenotypic analysis was performed by indirect immunofluorescence with fluorescence-conjugated goat anti-mouse Fab IgG as described previously⁴. Samples were analysed on an Epics V cell sorter. 10,000 cells were analysed in each sample. Each histogram displays the number of cells (ordinate) versus the intensity of fluorescence (abscissa) expressed on a logarithmic scale. Negative control used to determine background fluorescence was an ascites derived from a non-reactive hybridoma. Its reactivity is not shown on Fig. 1 since it was identical to that of anti-T4 for each line but JT7. For JT7 the reactivity of control ascites was identical to that of anti-T8. The monoclonal antibodies used in these studies have all been described in detail. T1 and T12 are expressed on a fraction of thymocytes and on a vast majority of peripheral blood T cells. However, anti-T1 defines a 67,000 molecular weight glycoprotein whereas anti-T12 defines a 120,000 molecular weight glycoprotein¹¹. Anti-T3 defines all mature T lymphocytes in human peripheral blood¹⁵ whereas anti-T4¹² and anti-T8¹⁶ are expressed on helper and cytotoxic/suppressor T cells, respectively. M01 is expressed on macrophages, polymorphonuclear leukocytes and LGL in peripheral blood¹⁷. The percentage of positive cells was always higher than 95% compared with less than 5% for anti-T1, anti-T3, anti-T4, anti-T8 and anti-T11. The reactivity of anti-T12 on JT3 was too weak to determine whether or not all the cells would express some antigen whereas others would be totally negative.

effector cells (data not shown). All the JT lines expressed both IA and T10 antigens (data not shown) which are known to be present on activated cells.

In parallel to these phenotypic characteristics, the lytic ability of the JT lines was investigated. The lines were tested against a variety of targets, either viral transformed or tumour cells, known to be sensitive to NK activity. Results presented in Table 2 are representative of at least three separate determinations, over a 5-week period of time for all lines except for JT7, which progressively lost NK activity. As expected, all the JT lines are able to kill K562 cells. However, JT4, JT6 and JT7 can only kill K562 whereas JT1, JT3, JT5, JT8 and JT9 are able to lyse additional targets. Moreover, these latter cell lines exhibit distinct reactivity. For example, Laz 221 is very sensitive to JT9 but not HL60, whereas JT1 significantly kills HL60 but not Laz 221.

The data presented above show a great diversity of both phenotype and lytic specificity within the JT lines. These lines display a unique and stable pattern of reactivity with six different monoclonal antibodies. However, only JT1, JT3 and JT9, which kill several targets, could be presently subcloned at 100 cells per well. All subclones tested so far displayed both the identical phenotype and functional activity of the original lines. Further studies will be necessary to determine if the efficiency of sub-cloning procedures can be enhanced by improving culture conditions. Nevertheless, this experimental approach allows the characterization of lymphocytes which are phenotypically distinct from known major populations of circulating mononuclear

cells and which are very likely to be present in low frequency in human peripheral blood. Alternatively, it can be argued that the culture conditions lead to the anomalous expression of cell-surface antigens on these cloned lines. However, these clones appear like large granular lymphocytes, kill target cells known to be sensitive to NK activity and, when tested⁴, can respond like normal human NK cells to interferon.

The present studies provide strong evidence for both phenotypic and functional heterogeneity of human cells capable of mediating 'natural killer-like' cytotoxicity to yet undefined antigens present on tumours or viral transformed cells. JT9 cells, for example, were found to display a phenotype similar to that observed on allogeneic CTL clones directed to class I antigens¹⁴. Although both expressed T3 and T8, JT9 was lytic against a variety of targets. Perhaps more importantly, the lytic activity of allogeneic T8+ CTL clones was blocked by either anti-T3 or anti-T8¹⁴, whereas the activity of JT9 on all targets could be blocked by anti-T3 only (data not shown). Nevertheless, since T3 is only expressed on a fraction of these NK lines, additional surface molecules must also be involved in these cytotoxic reactions. We believe that the identification of novel surface antigens will provide both additional insights into the lineage of many still undefined NK cells and a means for dissecting a structural basis of target cell recognition and lysis.

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A monoclonal antibody against antigen-specific helper factor augments T-cell help

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Antigen-specific molecules, commonly termed 'factors', have been shown to be released from helper and suppressor T cells^{1,2}. These factors mimic the activity of the cells that secrete them and there is much speculation about the relationship of antigen-specific factors to T-cell receptors for antigen¹⁻³. We have raised a variety of antisera in rabbits which were shown to react against conserved 'constant' determinants on either helper or suppressor factors independently of antigenic specificity or mouse strain of origin of the factor. In contrast, syngeneic mouse antisera were found to react with 'variable' factor determinants

in an antigen-specific and mouse strain-dependent manner⁴⁻⁸. These antisera thus define two regions on factor molecules, one 'variable' (related to antigen specificity) and the other 'constant' (related to function). However, potential contaminants in these antisera have limited their usefulness^{9,10}. Thus, we are now generating monoclonal antibodies against T-cell factors and report here the properties of a monoclonal antibody (AF3.44.4) which reacts with antigen-specific helper factors. This antibody also binds to helper T cells and, in the presence of antigen, augments helper cell induction *in vitro*, which, in turn, leads to enhanced antibody production *in vitro*. These characteristics suggest that AF3.44.4 recognizes a determinant shared by helper factor and the antigen receptor on helper T cells.

To produce monoclonal antibodies against 'constant region' determinants male Wistar rats were immunized with antigen column purified eluates of factor produced by 4-day incubation *in vitro* of mouse (DBA/2) spleen cells cultured with 100 $\mu\text{g ml}^{-1}$ of keyhole limpet haemocyanin (KLH)¹¹. These cultures have net suppressor function, but consist of mixtures of suppressor and helper cells^{10,12}, so that the antigen column-eluates used as immunogen contain both KLH-specific suppressor factor and KLH-specific helper factor. After repeated immunization with Sepharose-KLH purified factor, rat spleen cells were removed for fusion with the hypoxanthine-aminopterin-thymidine (HAT) sensitive NS-I myeloma using the method of Kohler and Milstein¹³ (see legend to Fig. 1).

Uncoloned hybrids were initially screened for rat immunoglobulin production by a solid phase radioimmunoassay using a ¹²⁵I-labelled monoclonal antibody specific for a rat κ light chain allotype marker (MRC OX12). Supernatants from cells found positive ($>1 \mu\text{g ml}^{-1}$) for rat immunoglobulin production were screened for their effect on *in vitro* secondary responses using trinitrophenylated (TNP)-KLH primed spleen cells over a 6-day culture period in the Mishell-Dutton culture system¹⁴. Those hybrid supernatants which augmented the antibody response were then cloned by limiting dilution¹⁵ and the cloned cells grown in nude mice to produce ascitic fluid which routinely contained 1-2 mg ml^{-1} antibody. All the experiments reported

here were performed with antibody purified by ion exchange chromatography¹⁶.

Clone AF3.44.4 (which produces an IgM, κ antibody) will enhance responses to several antigens, such as dinitrophenylated chicken globulin (DNP-CGG) or TNP-KLH (Fig. 1) and sheep red blood cells (SRBCs, data not shown). The activity is therefore dissociated from antigen specificity as was found with the anti-constant region activity of the rabbit anti-mouse factor antisera⁴. AF3.44.4 was raised against DBA/2 factor ($H-2^d$, $Igh-I^d$) but is not mouse strain specific as it affects *in vitro* responses from various strains tested such as CBA ($H-2^k$, $Igh-I^a$) (see Fig. 1) or C57BL/10Sn(B10) ($H-2^b$, $Igh-I^b$) (data not shown). Most significantly, the antibody is not a polyclonal activator of T cells as it will not stimulate primed cells in the absence of the appropriate antigen (Fig. 1).

To determine whether the enhancing properties of AF3.44.4 were due to inhibition of suppression or to augmentation of help, the antibody was bound to Sepharose beads and tested for its factor adsorption properties. The factors used in these experiments have been shown to be antigen specific¹¹ (Table 1). KLH-specific helper factor binds only to the inducing antigen (not to a control antigen, NP-bovine serum albumin (BSA)) and will provide help only in the presence of the inducing antigen (not to a control antigen, NP-GAT). As has previously been shown¹¹, these factors are not genetically restricted in their effect. AF3.44.4-Sepharose will bind helper factors regardless of their strain of origin or antigen specificity (expts a-c, Table 1) but does not bind suppressor factors (expt d, Table 1). Control rat IgM does not bind either helper or suppressor factors.

The immunological effects of AF3.44.4 were further analysed by *in vitro* KLH-specific helper and suppressor cell induction experiments using the Marbrook flask culture system^{11,17}. It was found that the antibody stimulated KLH-specific helper cell induction (Fig. 2a) but did not significantly affect the induction of KLH-specific suppressor cells (Fig. 2b). Thus augmented antibody responses were not mediated by blocking of T-cell suppression.

Table 1 Adsorption of helper factor by monoclonal antibody AF3.44.4 coupled to Sepharose beads

Expt	Factor†	Stimulus Antigen	Immunoabsorbent‡	Response (IgM PFC per 10^6 cells)§	Filtrate	Eluate
a	—	TNP-KLH	—	—	—	0
	BAB.14 HF _{KLH}	TNP-KLH	—	—	136 ± 24	—
	BAB.14 HF _{KLH}	TNP-KLH	Rabbit anti-HF	168 ± 27	—	44 ± 17
	BAB.14 HF _{KLH}	TNP-KLH	Rabbit anti-SF	19 ± 11*	—	166 ± 29
	BAB.14 HF _{KLH}	TNP-KLH	Rat IgM	210 ± 14	—	20 ± 10
	BAB.14 HF _{KLH}	TNP-KLH	AF3.44.4	10 ± 10*	—	177 ± 22
b	—	NP-GAT	—	—	0	—
	CBA HF _{NP}	NP-GAT	—	—	186 ± 17	—
	CBA HF _{NP}	NP-GAT	Rat IgM	197 ± 25	—	37 ± 13
	CBA HF _{NP}	NP-GAT	AF3.44.4	25 ± 4*	—	286 ± 10
c	—	TNP-KLH	—	—	6 ± 5	—
	B10.HTT HF _{KLH}	NP-GAT	—	—	11 ± 4	—
	B10.HTT HF _{KLH}	TNP-KLH	—	—	90 ± 2	—
	B10.HTT HF _{KLH}	TNP-KLH	KLH	15 ± 1*	—	86 ± 11
	B10.HTT HF _{KLH}	TNP-KLH	NP-BSA	69 ± 6	—	20 ± 6
	B10.HTT HF _{KLH}	TNP-KLH	Rat IgM	62 ± 12	—	18 ± 8
	B10.HTT HF _{KLH}	TNP-KLH	AF3.44.4	18 ± 9*	—	92 ± 8
	—	—	—	—	IgG PFC per 10^6 cells	
d	—	—	—	—	11 ± 19	—
	—	TNP-KLH	—	—	3,300 ± 165	—
	CBA SF _{KLH}	TNP-KLH	—	—	1,770 ± 215*	—
	CBA SF _{KLH}	TNP-KLH	Rat IgM	1,800 ± 753*	—	3,600 ± 380
	CBA SF _{KLH}	TNP-KLH	AF3.44.4	1,700 ± 190*	—	3,500 ± 715

In experiments a-c triplicate cultures of 3×10^6 unprimed CBA spleen cells were incubated with antigen (2 μg per well) and HF for four days before being collected and assayed for IgM plaque forming cells (PFC) by the Cunningham chamber method²⁹. In expt d triplicate cultures of 3×10^6 TNP-KLH primed CBA spleen cells were incubated with antigen (0.02 μg per well) and SF for 7 days before being collected and assayed for IgG PFC. HF, helper factor; SF, suppressor factor. * $P < 0.01$ as compared with controls. § The response in experiments a-c is shown as the IgM PFC per 10^6 initially cultured spleen cells $\pm$ s.e. and in experiment d as IgG PFC per 10^6 spleen cells $\pm$ s.e. † HF and SF were used at a final concentration of 2.5%. ‡ The factor adsorption and elutions, with acid-glycine (pH 2.8), were performed as previously described³⁰.

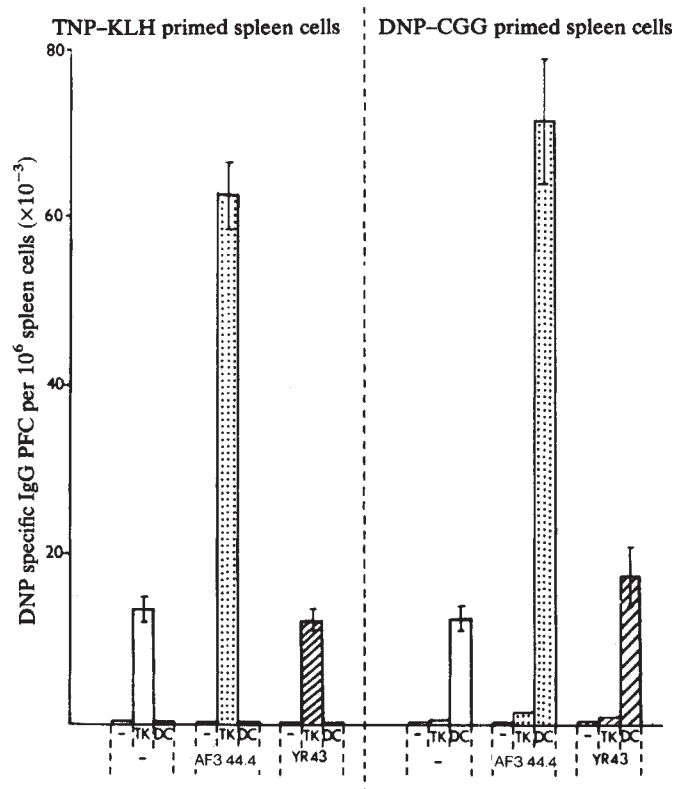


Fig. 1 CBA TNP-KLH primed spleen cells were cultured *in vitro* for 6 days at 5×10^5 cells per 200 μ l well in 96-well flat-bottomed Linbro plates with $0.05 \mu\text{g ml}^{-1}$ of antigen. Each group consisted of triplicate cultures, and the number of IgG DNP-specific PFC produced are expressed as PFC per 10^6 initially cultured spleen cells $\pm$ s.e. IgM plaques were inhibited with rabbit anti- μ antiserum. TNP-KLH primed spleen cells from CBA mice were cultured in the absence of antigen (-); in the presence of TNP-KLH (TK); or in the presence of DNP-CGG (DC). This was done in the absence of any antibody (-); in the presence of AF3.44.4 ($10 \mu\text{g ml}^{-1}$); or in the presence of control rat IgM, κ monoclonal antibody YR43 ($10 \mu\text{g ml}^{-1}$). The whole procedure was repeated with CBA DNP-CGG primed spleen cells, to demonstrate the lack of antigen specificity of the effect. The clone AF3.44.4 was produced in a fusion with the P3-NSI/1-Ag4-1 myeloma cell line and rat spleen cells by the method of Köhler and Milstein¹³. The immunogen was produced by DBA/2 spleen cells cultured in Marbrook-Diener flasks for 4 days with a high dose of antigen ($100 \mu\text{g ml}^{-1}$) and then subsequently for 24 h with a low dose of antigen ($0.1 \mu\text{g ml}^{-1}$). The supernatant from the 24-h culture was partially purified by elution from a KLH-Sepharose 4B column with Sorensen's acid glycine buffer, pH 2.8. Rats were injected with supernatants derived from 5×10^6 cells, initially in Freund's complete adjuvant (Difco), followed by three or four subcutaneous injections without adjuvant at 2-weekly intervals. Animals were rested for 4 weeks before the final boost (intraperitoneally), 4 days before fusion.

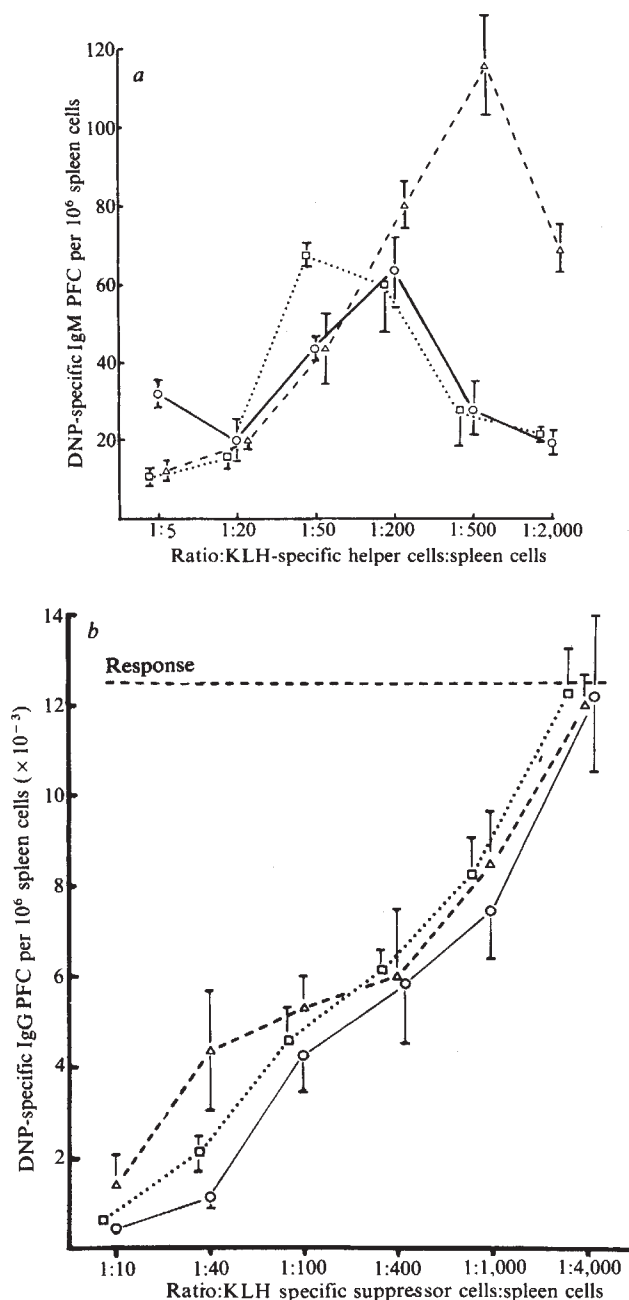


Fig. 2 *a*, The effect of monoclonal antibody AF3.44.4 on the induction of KLH-specific helper cells. Helper cells were induced in the absence of antibody (○—○), in the presence of AF3.44.4 at $10 \mu\text{g ml}^{-1}$ (△—△); or in the presence of a control rat IgM, κ antibody, YR43, at $10 \mu\text{g ml}^{-1}$ (□—□). *b*, The effect of monoclonal antibody AF3.44.4 on the induction of KLH-specific suppressor cells. Suppressor cells were induced in the absence of antibody (○—○); in the presence of AF3.44.4 at $10 \mu\text{g ml}^{-1}$ (△—△); or in the presence of YR43, a control rat IgM, κ antibody (□—□).

Methods: *a*, Helper cells to KLH ($\text{H}_{\text{C}}\text{KLH}$) were induced in Marbrook flasks by culture of unprimed spleen cells at 15×10^6 per ml in the presence of a low concentration of antigen¹⁷ ($0.5 \mu\text{g ml}^{-1}$ KLH). After 4 days, cells were collected, washed and passed over nylon wool columns to purify T cells before testing. Unprimed CBA spleen cells treated with monoclonal anti-Thy1.2 + C, were cultured for 4 days in a 200 μ l volume of RPMI + 10% fetal calf serum (FCS) with TNP-KLH ($0.1 \mu\text{g ml}^{-1}$) at 5×10^5 cells per well. Helper cells were titrated from a ratio of 1:5 to 1:2,000 (helper cells: spleen cells). Each group was set up in triplicate and assayed for PFC by the Cunningham chamber method²⁹ using DNP-Fab coated SRBCs and the background was subtracted using uncoated SRBCs. Results are expressed as the arithmetic mean PFC per 10^6 spleen cells $\pm$ s.e. In the absence of KLH-specific helper cells less than 10 PFC per 10^6 spleen cells were detected. *b*, Suppressor cells to KLH were induced in Marbrook flasks by a 4-day incubation of $15 \times 10^6 \text{ ml}^{-1}$ unprimed spleen cells with a high concentration of antigen¹¹ ($100 \mu\text{g ml}^{-1}$ KLH). After this period the cells were collected (recoveries being in the range of 30–50%), washed and passed over nylon wool columns, to purify T cells, before testing on CBA spleen cells from mice primed with TNP-KLH¹⁴. These were cultured for 6 days in a volume of 200 μ l with TNP-KLH ($0.05 \mu\text{g ml}^{-1}$) in flat bottomed microwells at 5×10^5 cells per well. Suppressor T cells were titrated from ratios of 1:10 to 1:4,000 (suppressor cells: spleen cells). Each group was performed in triplicate and assayed by the Cunningham chamber method for IgG DNP-specific PFC²⁹. Results are expressed as arithmetic mean PFC per 10^6 spleen cells $\pm$ s.e., and the response obtained in the absence of suppressor cells is shown by the dotted line. Response in the absence of antigen was less than 50 PFC per 10^6 spleen cells.

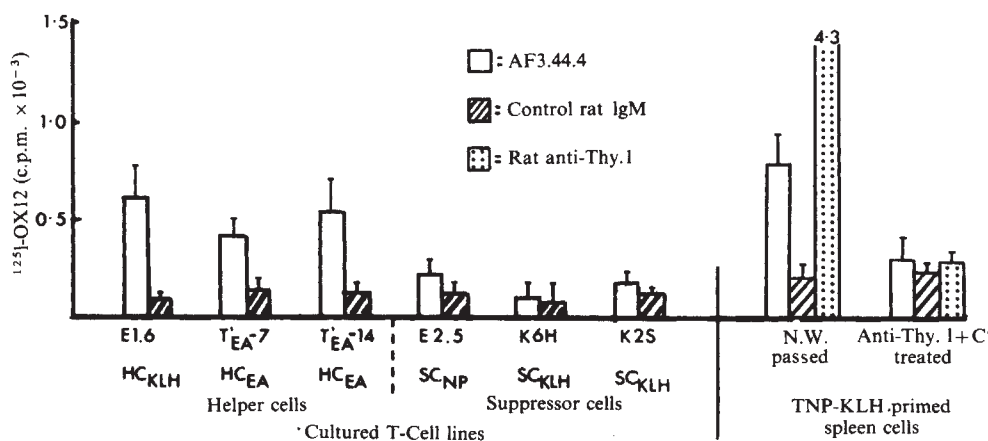
To determine the cell distribution of the determinant(s) defined by AF3.44.4, binding tests using various cultured T-cell lines were performed. Bound AF3.44.4 antibody was detected on the surface of T cells by using ¹²⁵I-labelled monoclonal anti-rat κ chain antibody (MRC OX12) (Fig. 3). The determinant(s) recognized by AF3.44.4 are expressed on T helper cell lines and tumours, but at low or undetectable levels on T suppressor cell lines (Fig. 3). By comparison anti-Thy.1 binding in this assay was at least five times greater, indicating that the AF3.44.4 antigen is expressed at low surface density.

In other experiments the binding of AF3.44.4 to antigen-primed spleen cells was tested (Fig. 3). Significant binding was observed when the cells were passed over nylon wool (T-cell enriched) but was removed by anti-Thy.1 + complement (C') treatment. Thus AF3.44.4 does not appear to bind to B cells or macrophages and other experiments have shown that cells from various other tissues (for example, heart, lung, liver, kidney) will not absorb the antibody.

The lack of antigen and strain specificity of AF3.44.4 was confirmed by the binding assays, as the antibody reacted with both KLH- and ovalbumin-specific helper cells derived from different strains of origin, that is, CBA $\times$ AKR-derived KLH-specific helper hybridoma (El.6) and B10-derived, ovalbumin-

Fig. 3 Cells were cultured for 2 h at 10^6 per well, in triplicate, with AF3.44.4 at $10 \mu\text{g ml}^{-1}$ or with control rat IgM at $100 \mu\text{g ml}^{-1}$ at 4°C in a volume of $100 \mu\text{l}$. After careful washing cells were incubated with ^{125}I -OX12 ($10\text{--}15 \mu\text{Ci} \mu\text{g}^{-1}$) containing 30,000 c.p.m. for 30 min at 4°C and then washed four times before gamma scintillation counting. The binding to the cultured T-cell lines of a monoclonal rat anti-Thy.1 antibody at a concentration of $1 \mu\text{g ml}^{-1}$ was in excess of 2.5×10^3 c.p.m. in the conditions used. Helper cells tested were E1.6, a T-cell hybridoma producing a KLH-specific helper factor³¹, and T_{EA}-7 and T_{EA}-14, two IL-2-dependent ovalbumin specific T helper cell clones provided by Dr M. Schreier.³²

The suppressor cells tested were E2.5, a T-cell hybridoma producing SF_{NP}³¹ and K6H and K2S, two radiation leukaemia virus induced T-cell tumours producing KLH-specific suppressor factor produced in collaboration with Dr P. Ricciardi-Castagnoli³³. In a separate experiment TNP-KLH primed spleen cells, either passed over nylon wool (to enrich for T cells) or anti-Thy.1 + C' treated (to enrich for B cells and macrophages), were tested to show specificity for T-cell binding. Fc binding was blocked by incubating the cells for 2 h at room temperature with medium containing 1% normal mouse serum.



specific interleukin-2 (IL-2) dependent helper cell clones (T_{EA}-7, T_{EA}-14).

As AF3.44.4 binds to helper cells and enhances helper cell induction but does not bind to suppressor cells or affect suppressor cell induction (Figs 2b, 3), it appears that AF3.44.4 does not enhance secondary responses by blocking the effects of suppression but by augmenting help in the presence of antigen. The way in which this occurs is not known in detail but it probably involves the direct activation of helper cells by AF3.44.4 and antigen, rather than enhancing the effects of helper factors. Preliminary evidence indicates that in the absence of helper cells, AF3.44.4 will inhibit responses generated by helper factors (unpublished data).

The capacity of AF3.44.4 to augment T-cell help, as well as absorb helper factors, is analogous to the effects of anti-immunoglobulin antibodies. Although anti-immunoglobulin antisera will bind secreted immunoglobulin they can also activate B cells to divide^{18,19}, and release more antibody²⁰ by acting on immunoglobulin at the cell surface. In addition, anti-idiotypic antisera have been shown to activate T-cell clones, inducing proliferation and the release of IL-2 (ref. 21) and, in other systems, anti-idiotypic antibodies will mimic the effects of antigen²².

There is evidence that T cells and antigen-specific factors react with antisera that recognize the heavy chain variable region (V_H) of immunoglobulin molecules²³⁻²⁵ and it has been proposed that the constant region of T-cell-derived antigen recognition molecules, although distinct from those of B-cell immunoglobulin heavy chains (Igh), may be linked to the Igh locus^{7,8,26}. Owen and Spurlin²⁶⁻²⁸ have shown that polyclonal antisera or monoclonal antibodies raised in Igh-1 allotype congenic mice define subsets of T cells which can be activated polyclonally with the antisera. However, there is as yet no direct evidence that these determinants are expressed on the antigen-specific receptors or factors of T cells.

The monoclonal antibody reported here reacts with helper factors and with a surface determinant on helper cells. The latter is a candidate for the antigen-specific receptor on helper cells as suggested by the way in which AF3.44.4 activates helper cell in the presence of antigen. It provides a new method for analysing the mechanisms of helper T-cell induction and may provide a potential probe for the identification and analysis of the elusive T-cell receptor for antigen. The augmentation of T-cell help by a monoclonal antibody also provides a new method of regulating immune responses.

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T-system optical signals associated with inward rectification in skeletal muscle

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The resting potential of many excitable cells, including skeletal muscle¹⁻⁴, cardiac muscle^{5,6}, nerve cell bodies⁷ and egg cells⁸, is determined by a resting potassium conductance which shows inward rectification, allowing potassium ions to move more readily inward across the cell membrane than outward. In skeletal muscle, where inward rectification has been extensively studied, a large part of this conductance is located in the T-system membranes^{2,3,9-11}. However, to date, only the kinetic and voltage-dependent properties of this conductance have been studied from analyses of the membrane potential or current recorded at the fibre surface. We report here measurements, obtained using a voltage-sensing dye, of potential changes in the T-system membranes associated with the inwardly rectifying K⁺ current. Our results show that this conductance alters the time course and significantly attenuates the amplitude of the potential change across the tubular membranes. These optical data provide new evidence for the presence of this conductance in the T-system and, when analysed using a radial cable model for the T-system, provide an estimate of the distribution of the inward rectifier conductance over the surface and T-system which is in agreement with estimates obtained by other techniques.

Inward potassium currents and optical signals were recorded simultaneously from single semitendinosus fibres of *Rana catesbiana* exposed to high potassium Ringer's and mounted in a three-Vaseline-gap voltage-clamp set-up¹² modified for optical recording^{13,14}. Potential changes across the T-system membranes were monitored with the non-penetrating potentiometric dye NK2367 (refs 14, 15) using unpolarized light at 670 nm. The dye-related absorbance change recorded in these illuminating conditions arises predominantly in the T-system and is believed to represent a 'weighted average' of changes in tubular potential¹⁴. In control experiments, a surface membrane signal¹⁴ (which closely follows the applied voltage step) was recorded at 720 nm, suggesting that the wavelength dependence of the optical signals previously reported for this dye¹⁴ was not altered by our experimental conditions.

Figure 1a shows the membrane potential (top traces), current (centre traces) and optical signals (lower traces) recorded for voltage-clamp steps of -80 mV (left) and +80 mV (right) from the holding potential. The membrane current shows a pronounced inward rectification, being about nine times larger for the hyperpolarizing than for the depolarizing pulse. The magnitude of the tubular optical signals (lower traces) also shows a marked asymmetry, in this case being smaller for the hyperpolarizing pulse. In addition, neither the hyperpolarizing nor depolarizing optical signals have a time course which follows the potential step imposed across the surface membrane (top traces), which in this preparation is voltage-clamped to a steady level within 20 μ s (ref. 12). Both rise to a maximum value less rapidly than the imposed potential step, and are followed by a small, slow decline and an undershoot of the signal at the 'off' of the pulse. After the addition of Cs (10 mM) and tetraethylammonium (TEA, 50 mM), which block inward rectification^{16,17}, the inward current is significantly reduced (>90%), and both the current and optical records become more symmetrical in

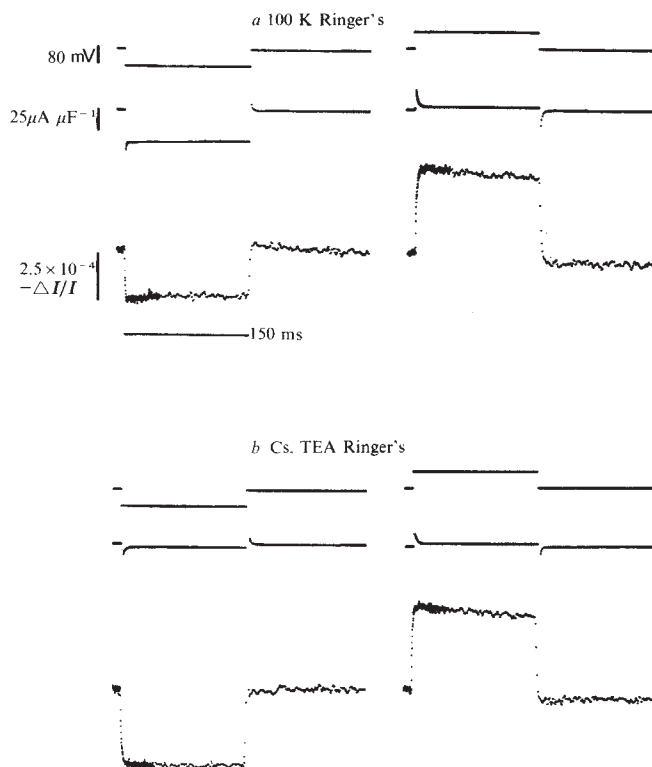


Fig. 1 Membrane potential (upper traces), membrane current (centre traces) and optical signals (lower traces) recorded for voltage steps of -80 mV (left) and +80 mV (right) from the holding potential (-25 mV). *a*, 100 K Ringer's; *b*, Cs, TEA Ringer's. The optical traces in *b* were scaled using the ratio of the peak optical signals at +60 mV before and after the solution change (scaling factor = 1.12), to correct for a small loss of signal due to dye bleaching^{14,15}. The fibres were dissected, mounted and voltage-clamped following procedures described elsewhere^{12,14}. Membrane current is expressed as the total current divided by the total capacitance recorded from the fibre segment in the A-pool (recording pool, see ref. 12). The holding potential was set to -5 mV in most fibres (V_K), but was -25 mV for the fibre shown here and in Fig. 2a, b. The external solution for the traces shown in *a* (100 K Ringer's) contained: K₂SO₄ (50 mM), Ca₂SO₄ (8 mM), Tris-HEPES (5 mM, pH = 7.4); the external solution for the traces in *b* (Cs, TEA Ringer's) contained: K₂SO₄ (50 mM), TEA₂SO₄ (25 mM), Cs₂SO₄ (5 mM), Tris-HEPES (5 mM, pH 7.4); the internal solution contained: K₂SO₄ (50 mM), K-MOPS (20 mM), Tris-EGTA (30 mM), Na₂ATP (5 mM), MgSO₄ (5 mM), Na₂PCr (5 mM). pH = 7.1. All solutions were adjusted to a final osmolarity of 300 mOsm with sucrose. The fractional transmittance change, $\Delta I/I$ (where ΔI is the intensity change elicited on stimulation and I is the resting intensity) was detected from a recording area equal to the fibre diameter times the A-pool width (130 $\times$ 150 μ m). The fibre segment in the A-pool was stained for 15 min with 0.5 mg ml⁻¹ NK2367 dye in the external solution, and illuminated with unpolarized, monochromatic light at 670 nm. After staining, a concentration of 0.06 mg ml⁻¹ was kept in the external solution. The data were acquired digitally and signal-averaged¹⁴. The time constant of the optical recording system in response to an LED light pulse was 10 μ s. An additional anti-aliasing filter (6-pole Bessel, low pass) in front of the A/D was set to the Nyquist frequency calculated for the sampling interval. Baseline subtraction was performed for the optical traces¹⁴. Two sweeps averaged per trace. Fibre diameter, 130 μ m; sarcomere spacing, 2.16 μ m; fibre capacitance, 7.4 μ F cm⁻²; temperature, 10 $^{\circ}$ C.

amplitude (Fig. 1b). The slow decline and undershoot in the hyperpolarizing optical signal also disappear, but the time course of the depolarizing optical signal is unchanged. These results suggest that the attenuation of the hyperpolarizing optical signals recorded in 100 K Ringer's solution is related to the presence of a Cs-sensitive, inward potassium conductance in the T-system. The slow decline and undershoot observed in

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Fig. 2 *a*, Voltage-dependence of inward rectification, plotted for the same fibre as Fig. 1. 100 K Ringer's (○); Cs, TEA Ringer's (●). For each set of data a small linear leak conductance of 0.019 ms per μF was subtracted. Arrow indicates the holding potential. The shape of the current-voltage relation in Cs, TEA Ringer's results from the voltage-dependence of the block by Cs^+ (ref. 17). *b*, Voltage-dependence of the optical signals recorded from the same fibre. 100 K Ringer's (○), TEA Ringer's, Cs (●). The straight line is the linear regression fitted to the data at positive potentials in both solutions, from the equation, $-\Delta I/I \times 10^4 = 0.056 V_m - 0.012$ (correlation coefficient 0.99). No bleaching correction was used for the set of points taken in 100 K Ringer's as the change in $\Delta I/I$ during this set of measurements was less than the r.m.s. optical noise (0.2×10^{-4}). Similarly, during the measurements in Cs, TEA Ringer's, no significant bleaching occurred. However, in the interval between the two sets of measurements, some loss of signal occurred and the set of data recorded after the change to Cs, TEA Ringer's was scaled using the +60 mV signals in both solutions as the scaling reference (scaling factor 1.12). *c*, Attenuation of the optical signals at negative potentials recorded in 100 K Ringer's, from five fibres, expressed as a % of the peak optical signal recorded for the equivalent depolarizing pulse. Different symbols represent different fibres. Mean fibre diameter, $156.6 \pm 10.2 \mu\text{m}$ ($n = 5$); mean sarcomere length, $2.01 \pm 0.07 \mu\text{m}$ ($n = 4$); mean capacitance, $8.24 \pm 0.82 \mu\text{F cm}^{-2}$ ($n = 5$).

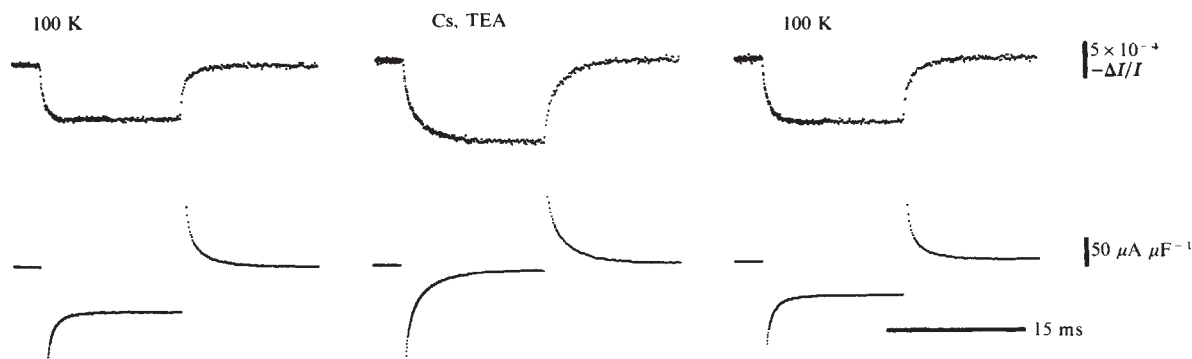
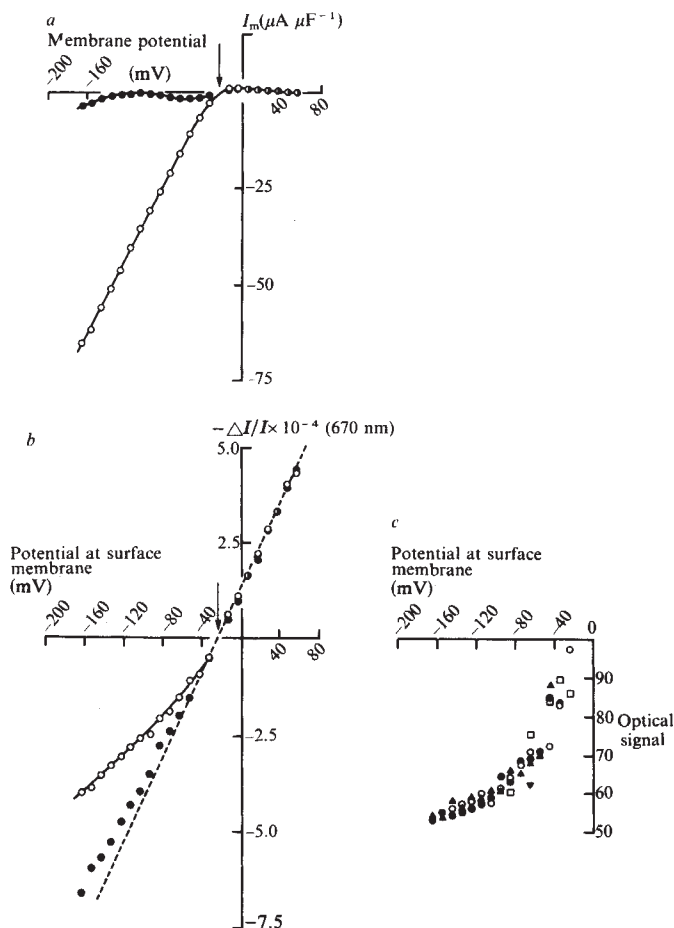


Fig. 3 Optical signals (upper traces) and membrane current (lower traces) for a 15-ms pulse to -120 mV recorded in 100 K Ringer's (left), in Cs, TEA Ringer's (centre), and after return to 100 K Ringer's (right). Holding potential, -5 mV (V_K). The sampling interval was 20 μs per point for the first 512 points and 50 μs per point for the second 512 points. The optical signals recorded after each solution change were scaled using the ratios of the corresponding +60 mV depolarizing optical signals before and after the solution change as the scaling reference (scaling factors 1.25 and 1.32). Four sweeps averaged per trace. Fibre diameter, $135 \mu\text{m}$; sarcomere spacing, $2.1 \mu\text{m}$; fibre capacitance, $7.6 \mu\text{F cm}^{-2}$; temperature, 10°C .

the optical signals may be related to kinetic properties of membrane conductances (because it is reduced by Cs^+), but may also be a slow dye-related second component¹⁸. Because of the small amplitude and slow time course of these phenomena, they do not significantly affect the peak value of the optical signal.

The voltage-dependence of the peak optical signal and the peak inward current are compared in Fig. 2*a, b*. In 100 K Ringer's solution, outward currents are very small, and the depolarizing optical signals are linearly dependent on the potential imposed across the surface membrane. At potentials more negative than -30 mV , where there are substantial inward currents, the peak amplitude of the optical signals is significantly smaller than that recorded at positive potentials, and deviates

from the straight line fitted through the depolarizing optical data. After the addition of Cs^+ and TEA^+ , however, the inward current is almost completely blocked and the optical data at hyperpolarizing potentials approach the fitted line. This suggests that the less steep voltage dependence of the hyperpolarizing optical signals recorded in 100 K Ringer's solution is caused by the activation of the inward rectifier. This change would be expected if some fraction of this conductance, located in the T-system, caused a decrease in potential sufficient to prevent the tubular membranes from being voltage-clamped to the potential imposed at the surface membrane.

Figure 2*c* shows the per cent attenuation in the amplitude of the hyperpolarizing optical signals, measured over the same range of potentials, in five fibres. In all fibres, this attenuation

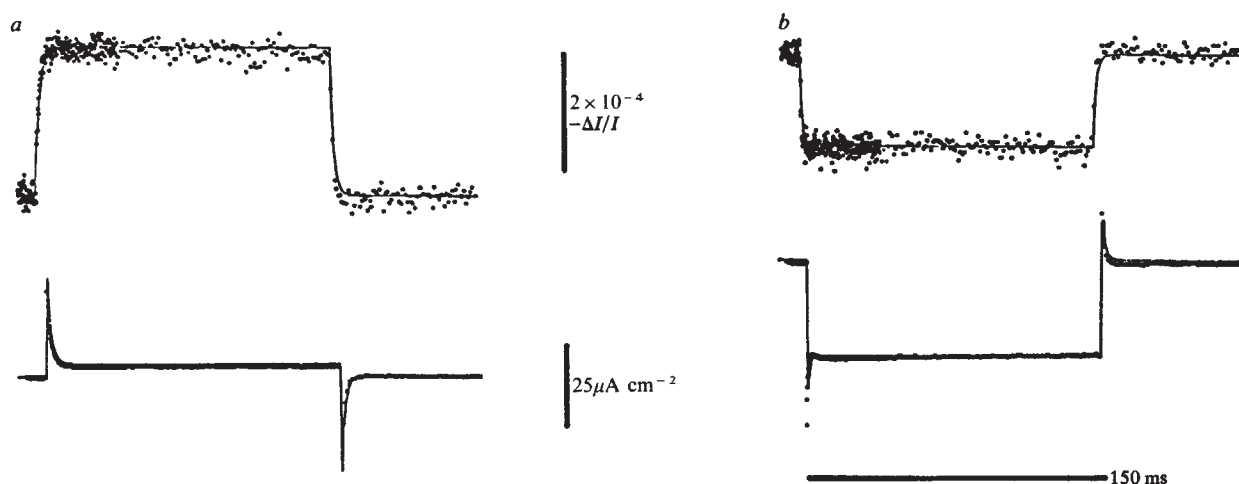


Fig. 4 Predicted (—) and measured (●) curves for the average T-system potential change and the total (radial + surface) membrane current, for voltage-clamp pulses of +60 (a) and -60 (b) mV from V_K (-5 mV). Theoretical curves for the membrane current were computed numerically from equation (9) of Adrian *et al.*²⁰, solved for the boundary conditions of a voltage-clamped fibre and including an access resistance at the mouth of the tubules^{20,21}. Theoretical curves for the optical data are taken from the radial integral of the tubular potential computed from this equation. In this integration, we made the simplifying assumption that the intensity change per mV per unit membrane area is constant along the radius, although tests of this assumption have not yet been performed. The radial cable equation was solved numerically using an implicit Crank-Nicholson algorithm and a gaussian elimination method^{22,23}, for a radial cable consisting of 15 segments. The total ionic current was computed by adding the surface and T-system contributions, assuming that a small ohmic leak conductance and a voltage-dependent conductance of the form $g = \bar{G}(V - V_K)/(1 + \exp[(V - V')/k])$ (refs 8, 24), where $\bar{G}$ = the specific membrane conductance (per unit area of membrane), V = membrane potential, V' = the potential of half-maximal $\bar{G}$, and k = a steepness factor. The basic constants used for the theoretical curves (following the definitions given in ref. 20) are: fibre diameter, 130 μm ; access resistance, $50 \Omega^{-1} \text{cm}^2$; lumen conductivity, $8.7 \times 10^{-3} \Omega^{-1} \text{cm}^{-1}$; leak conductance, $4 \times 10^{-5} \Omega^{-1} \text{cm}^2$; specific potassium conductance of the surface and T-system, $0.55 \times 10^{-3} \Omega^{-1} \text{cm}^2$; specific membrane capacitance of surface and T-system, $1 \mu\text{F cm}^{-2}$; k , 10; V' , -30 mV. The geometrical constants used are those given in ref. 20. The optical data shown are taken from a fibre of 135 μm diameter.

increases at more negative potentials and approaches a value $\sim 55\%$ of the equivalent depolarizing optical signal at -140 mV (mean fibre diameter $156.6 \pm 10.2 \mu\text{m}$). If we assume that the 670 nm optical signal represents a 'weighted average' of potential changes occurring along the entire fibre radius¹⁴, this result implies that when the inward rectifier is activated, the 'weighted average' tubular membrane potential in these fibres reaches a value of approximately -60 mV for a voltage-clamp pulse of -100 mV.

Figure 3 shows optical (upper traces) and current (lower traces) records taken using a faster sampling interval and short pulses, to analyse more fully the effect of this conductance on the speed of the tubular potential change. In 100 K Ringer's solution, for a fast potential step of +120 mV, a large inward current is present and the tubular optical signal rises to 67% of the maximum value in 0.52 ms, at 10 °C. In Cs, TEA Ringer's, the inward current is reduced by 90% and the optical signal rises to 67% of the maximum value in 1.2 ms. These effects of Cs^+ and TEA^+ on both the current and optical signals were almost completely reversible; the inward current recovered by 80%, and the time constant of the optical signal was 0.60 ms. As expected, the time courses of the depolarizing optical signals (not shown) were not significantly changed by the Cs, TEA solution.

The results described above suggest that the inward rectifier conductance has significant effects on both the amplitude and time course of potential changes occurring in the tubular membranes. The optical data thus provide independent evidence that some fraction of the inward rectifier channels is located in the tubular membranes. A more quantitative description of the radial decrease in the tubular potential can be obtained if the optical signals are modelled assuming a particular equivalent circuit for the T-system. We have made a preliminary attempt to do this using a radial cable model¹⁹⁻²¹ for the tubular network. Because the optical signals are recorded from the entire T-system, the radial integral of the predicted tubular potential was taken to model the optical signals. The results of this analysis are shown in Fig. 4. In both the surface and T-system, we included a voltage-dependent, instantaneously activating,

saturable conductance. With a simple model of this form, both the total measured current (radial + surface) and the time course and attenuation in the tubular optical signal were simultaneously predicted. In this fibre, the predicted 'weighted average' potential change elicited for a -80 mV pulse was -47 mV, and the decremented tubular potential change at the centre of the fibre was -31 mV. Our data fit reasonably well with the assumption of an equal distribution of inward rectifier conductance over the surface and T-system. For this distribution, the predicted tubular fractional contribution to the total measured current was 83% at -80 mV, for a fibre of 130 μm diameter. This distribution is similar to that previously estimated from the decline in inward rectifier current due to depletion of K^+ from the tubular lumen^{3,11}.

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Evolutionary divergence of the mRNA transcription initiation mechanism in yeast

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The promoters of eukaryotic genes are being increasingly defined through the identification of consensus DNA sequences, by mutational analysis, and by *in vitro* and *in vivo* studies of transcription¹⁻⁴. Whereas the TATA sequence (Goldberg-Hogness box) has been largely conserved among protein encoding genes (transcribed by RNA polymerase II) of eukaryotes^{5,6}, there is some evidence that other structural and functional determinants of mRNA transcription are not conserved between species⁷⁻⁹. I report here an *in vivo* comparative analysis of the transcription initiation systems of the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* (which can both be transformed by identical plasmids¹⁰). I have found no instance in which a gene is transcribed in the same fashion in both yeasts. Instead, I have found that the *in vivo* transcription starting points for many different yeast genes are determined by the cell in which it is transcribed rather than its gene structure alone. The evidence also suggests that the divergence of the transcription initiation system may partly involve the mechanism or structure which determines the distance from the TATA consensus sequence to the site of transcription initiation.

Investigations of the transcription of the *S. pombe* cytochrome *c* gene (*CYC*)¹¹ in *S. cerevisiae* first indicated that the RNA polymerase II transcription initiation mechanisms of *S. pombe* and *S. cerevisiae* are functionally different. RNA transcribed from the *S. pombe* *CYC* gene [on the plasmid YRp7PoCYC(4.3) in *S. pombe* and *S. cerevisiae*] was analysed by S₁ mapping¹² (Figs 1, 2). The *S. pombe* transcripts start at positions -119 and -112 (the A of the translation initiation codon ATG being designated position +1), ~36 nucleotides downstream from the centre of an A+T-rich sequence which bears resemblance to the Goldberg-Hogness box. The *S. cerevisiae* transcripts do not coincide with the *S. pombe* start sites. A large fraction of the *S. cerevisiae* transcripts start before the 3' end of the DNA probe at -140. Specific 5' ends can be seen downstream of the *S. pombe* start sites, at positions -37 and -47, ~115 nucleotides from the TATA region. This observation was interesting in light of the fact that in *S. cerevisiae* the distance between the TATA sequence and the transcription start site is often approximately 100 nucleotides⁶.

The *S. pombe* alcohol dehydrogenase (*ADH*) gene¹³ contains a sequence starting at position -113 which is perfectly homologous to the Goldberg-Hogness sequence TATAAATA and also to sequences located about 100 nucleotides upstream of the transcript start sites of the two *S. cerevisiae* *ADH* genes^{14,15}. The major homologous transcript start site of the *S. pombe* *ADH* gene is at -66 (47 nucleotides from TATAAATA), and a minor start site is 4 nucleotides further downstream (Figs 1 and 2). This gene is transcribed in a radically different fashion when present in *S. cerevisiae* cells, carried on the plasmid pADHpol. There are no *S. cerevisiae* transcripts that coincide with the two *S. pombe* transcripts (Figs 1, 2). A significant fraction of the *S. cerevisiae* transcripts start upstream of the 3' end of the S₁ probe at approximately position -300. The *S. cerevisiae* transcripts starting downstream of -300 are very heterogeneous with respect to their 5' end. The most prevalent transcript initiation site is at approximately position -20, 97 nucleotides downstream from TATAAATA, and the

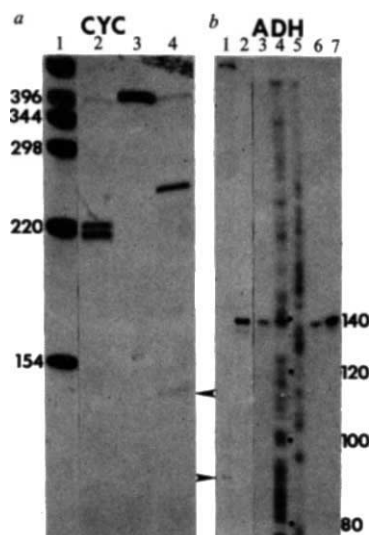


Fig. 1 S₁-nuclease mapping of *S. pombe* *CYC* and *ADH* transcripts made in *S. pombe* and *S. cerevisiae*. The figure shows autoradiograms of the 8% polyacrylamide sequencing gels from which the sizes of the S₁ protected DNA were determined. The locations of heterologous transcripts starting downstream from TATA are indicated by arrows. The lanes are identified below with respect to the cells from which the RNA was extracted and the carbon source in the medium in which the cells were grown. **a**, Transcript mapping of *S. pombe* *CYC* gene. Lane 1, DNA size standard-*Hinf*I digested pBR322 labelled with [α -³²P]dCTP using Klenow polymerase; lane 2, *S. pombe* 972 h⁻, glucose; lane 3, intact probe; lane 4, *S. cerevisiae* GM3C-2¹⁶ transformed with YRp7PoCYC(4.3), glucose (the major 247-nucleotide band represents complete protection of the *S. pombe* derived portion of the probe). **b**, Transcript mapping of *S. pombe* *ADH* gene. Lane 1, *S. cerevisiae* 500-11¹³ transformed with pADHpol, glucose; lane 2, *S. pombe* 972 h⁻, glucose; lane 3, *S. pombe* 972 h⁻, glucose; lane 4, Maxam and Gilbert (A+G) reaction using probe; lane 5, Maxam and Gilbert (C+T) reaction using probe; lane 6, *S. pombe* 972 h⁻, glycerol; lane 7, *S. pombe* *leu1-32* transformed with pADHpol, glucose.

Methods: RNA isolation, poly (A)⁺ RNA enrichment, and S₁-nuclease mapping was performed as previously described¹¹. Transformation of *S. pombe* *leu1-32* using YEpl3-¹⁹ derived plasmids was accomplished using the *S. cerevisiae* transformation procedure^{20,21} with the following modifications. After a 10-min incubation at 30°C in SED (1 M sorbitol, 0.025 M EDTA, pH 8, 0.05 M dithiothreitol) cells were washed in 1 M sorbitol and resuspended in SCE (1 M sorbitol, 0.1 M sodium citrate, pH 5.8, 0.01 M EDTA)/1% Glusulase (Endo). After a 30-min incubation at 30°C, Zymolyase 60,000 (Kirin Brewery Co.) was added to a concentration of 1 mg ml⁻¹. After a 7-min incubation at 30°C the cells were pelleted and washed in 1 M sorbitol. At this stage 50–90% of the cells lysed when placed in water. The transformation frequency was typically about 2 × 10⁻³ transformants per regenerated protoplast. By hybridizing total poly(A)⁺ RNA to 5' end-labelled separated strands of restriction fragments that cover the 5' end of the gene and adjacent sequences, and then measuring the lengths of the ³²P DNA strands protected from S₁ digestion, the distance from each RNA terminus to a restriction site has been measured. 25 µg of poly(A)⁺ RNA was used in each hybridization. The probe used in **a** was made by strand separating the 5' end-labelled 391-nucleotide *Ava*II fragment of pPoCYC(0.54)¹¹, which extends from +103 to -144 and includes a further 144 nucleotides derived from pBR322 at the 3' end. The faster-migrating strand is complementary to mRNA. The probe used in **b** was made by cutting the ~1.75-kb *Bgl*II (+876)–*Sal*I (–900) fragment of pADHpol¹⁴ with *Taq*I, labelling with [γ -³²P]ATP, cutting again with *Hpa*II, and purifying the single-stranded 407-nucleotide 5' end-labelled *Taq*I (5', +107)–*Hpa*II (3', –300) fragment on a strand separation gel.

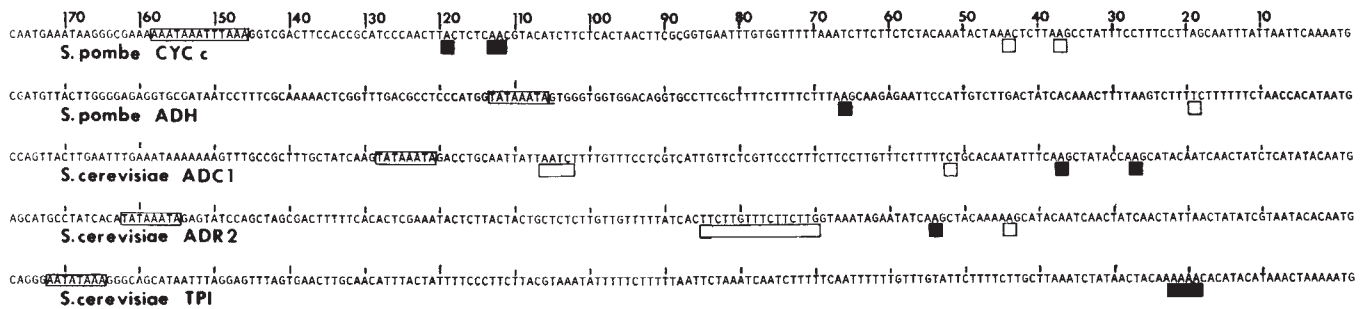


Fig. 2 Homologous and heterologous transcription initiation sites of *S. pombe* and *S. cerevisiae* genes. Shown are the 5'-flanking regions extending from -177 to +3. The homologous start sites are represented by full boxes under the sequence, the heterologous start sites (*S. cerevisiae* transcripts of *S. pombe* genes, and *S. pombe* transcripts of *S. cerevisiae* genes) are represented by empty boxes. Sequences best matching the Goldberg-Hogness box (TATAAATA) are boxed. The *TPI* gene contains two sequences that closely match TATAAATA starting at positions -170 and -107. The sequence at -170 is boxed because it is flanked by purine residues, which seem to be preferred around most presumed yeast TATA boxes. The references for the DNA sequences are given in the text.

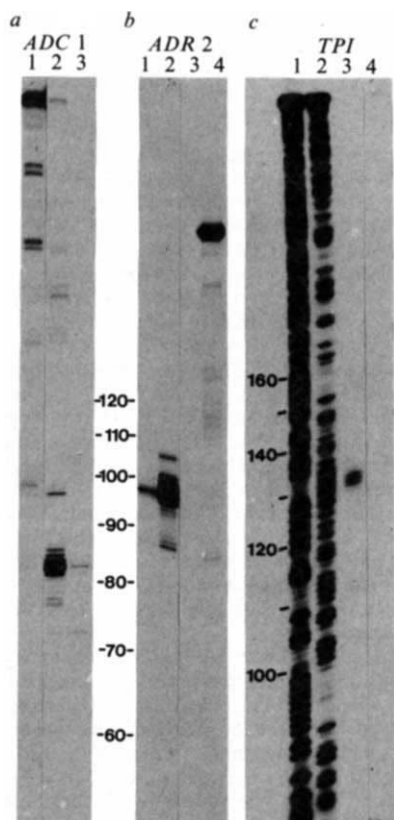


Fig. 3 S₁-nuclease mapping of the *S. cerevisiae* and *S. pombe* transcripts of the *S. cerevisiae* *ADC1*, *ADR2*, and *TPI* genes. The figure shows autoradiograms of the S₁-nuclease mapping experiments. The lanes are described below with respect to the cells from which the RNA was extracted, the medium in which the cells were grown, and the amount of poly(A)⁺RNA used in the hybridization. **a**, Transcript mapping of *S. cerevisiae* *ADC1* gene. Lane 1, *S. pombe* leu1-32 transformed with pADC1-2C, glucose, 25 µg; lane 2, *S. cerevisiae* AB320, glycerol and ethanol, 25 µg; lane 3, *S. cerevisiae* AB320, glucose, ~1 µg. **b**, Transcript mapping of *S. cerevisiae* *ADR2* gene. Lane 1, *S. cerevisiae* AB320, glycerol and ethanol, 5 µg; lane 2, *S. cerevisiae* AB320, glycerol and ethanol, 25 µg; lane 3, *S. cerevisiae* AB320, glucose, ~1 µg; lane 4, *S. pombe* leu1-32 transformed with pADR2-2L, glycerol and ethanol, 25 µg. **c**, Transcript mapping of *S. cerevisiae* *TPI* gene. Lane 1, Maxam and Gilbert purine (A+G) reaction using probe; lane 2, Maxam and Gilbert pyrimidine reaction (C+T) reaction using probe; lane 3, *S. cerevisiae* AB320, glucose, ~1 µg; lane 4, *S. pombe* leu1-32 transformed with pTPI-10, glucose, 25 µg. **Methods:** The probe used in **a** was made by cutting the 1,174-bp *Hind*III (+764)-*Sph*I (-40) fragment of pADC1-2C with *Hin*FI, 5'-end-labelling with [γ -³²P]ATP, and isolating the 453 nucleotide *Hin*FI (5', +43)-*Sph*I (3', -410) fragment, which migrates faster than its complement. The probe used in **b** was made by cutting the 937-bp *Hind*III (+764)-*Sph*I (-173) fragment of pADR2-2L with *Hin*FI, 5'-end-labelling with [γ -³²P]ATP, and isolating the 216 nucleotide *Hin*FI (5', +43)-*Sph*I (3', -175) fragment, which migrates faster than its complement. The probe used in **c** was made by cutting the 1.2-kb *Bgl*II (+297)-*Bgl*I (-900) fragment from the plasmid pTBR²² with *Taq*I, 5'-end-labelling with [γ -³²P]ATP, cutting with *Sph*I and isolating the 33-bp *Taq*I (5', +109)-*Sph*I (3', -224) fragment.

majority of the other transcripts start within 15 nucleotides of this site.

These results indicate that the *S. cerevisiae* and *S. pombe* transcription initiation mechanisms have diverged. The argument could be made, however, that the differences in transcription result from the fact that the heterologous genes were carried on plasmids, and that in such conditions the genes would not be properly transcribed even in their hosts. To rule out this possibility, I examined the *ADH* transcripts from *S. pombe* leu1-32 cells that had been transformed with pADHpol. The pattern of S₁ nuclease protection was the same in the transformed cells as in the untransformed cells (Fig. 1). The level of *ADH* mRNA is three to five times higher in the transformants, so it is assumed that the majority of the *ADH* mRNA is transcribed from the plasmid-borne gene.

Two lines of evidence suggest that the transcription initiation mechanisms of the two yeast differ in a spacer element that

determines the distance from the TATA box to the 5' end of the mRNA. First, the distance from TATA to start is significantly less in the two fission yeast genes examined than in the similar budding yeast genes *CYC1*¹⁶, *ADC1*¹⁴ and *ADR2*¹⁵. Second, the distance seen in the *S. cerevisiae* transcripts of *S. pombe* genes is typical of that for *S. cerevisiae* genes transcribed homologously. If the spacer element is actually longer in budding yeast than fission yeast, it might be expected that the *S. pombe* transcripts of *S. cerevisiae* genes would start upstream of the *S. cerevisiae* start sites. To test this hypothesis, I compared the *S. cerevisiae* and *S. pombe* transcripts of the *S. cerevisiae* genes *ADC1*, *ADR2* and *TPI*.

It has been previously reported¹⁴ that the *S. cerevisiae* transcripts of the *ADC1* gene, which encodes glucose constitutive alcohol dehydrogenase isozyme I (ADHI), start at -27 and -37, 101 and 91 nucleotides downstream from the sequence TATAAATA. My results are in basic agreement with this,

showing that the -37 sequence is preferred and revealing an additional minor start site at -52 (Figs 2, 3). The intact *ADC1* gene, along with 4 kilobases (kb) of 5' flanking sequence, was transformed into *S. pombe* *leu1-32* on the plasmid pADC1-2C. The *S. cerevisiae* start sites at -27 and -37 are not used in *S. pombe*, with virtually all transcription starting upstream of these sites (Fig. 3). The majority of the transcripts start upstream of the 3' end of the DNA probe at -410, although significant transcription does start slightly upstream of the minor *S. cerevisiae* start site at -52. Similar transcript mapping results were obtained when the *ADC1* promoter fused to a cDNA copy of the rat growth hormone gene was introduced into the two yeasts (G. Ammerer, personal communication).

The homologous transcripts of the *S. cerevisiae* *ADR2* gene, which encodes the glucose-repressible isozyme ADHII, have been shown to start at around -55, 107 nucleotides downstream from TATAATA¹⁵. I have obtained the same result, and have also detected minor transcripts starting at -45 and -64 (Figs 2, 3). The transcription pattern of this gene when present in *S. pombe* on the plasmid pADR2-2L is very different, with almost no initiation at -55 and very low levels at the minor *S. cerevisiae* start sites. The majority of the *S. pombe* transcripts of *ADR2* start upstream of the 3' end of the *S₁* probe at -175. As was the case for *ADC1*, the majority of the *S. pombe* transcripts which did not fully project the probe started closer to the TATA box than did the *S. cerevisiae* transcripts, in this case in the -70 to -85 region.

I have found that the major homologous transcripts of the *S. cerevisiae* *TPI* gene¹⁷, which encodes triose phosphate isomerase, start at approximately -20, 140 nucleotides downstream from the sequence TATAAAGG (Figs 2, 3). I introduced the *TPI* gene along with ~3.5 kb of 5' flanking sequence into *S. pombe* on the plasmid pTPIC-10. No transcription initiating near the beginning of the *TPI* gene was detected in *S. pombe*. Instead only a low level of RNA starting before the 3' end of the probe at position -224 was detected. This result is very curious, since the *TPI* gene is one of the most highly transcribed genes in *S. cerevisiae* and therefore is presumed to have a very strong promoter. A possible explanation for this observation may be the lack in *S. pombe* of an equivalent of the *S. cerevisiae* gene *GCR* whose product appears markedly to promote expression (probably by enhancing transcription) of genes which encode glycolytic enzymes such as *TPI*¹⁸.

The results described here, and those of others⁷⁻⁹ lead to the conclusion that, at least in functional terms, the eukaryotic RNA polymerase II transcription initiation mechanism has not remained a static element during evolution. The exact nature of the functional divergence is unclear; however, my results suggest that, in part, it involves the mechanism that determines the distance from the TATA box to the site of transcription initiation. The strongest evidence for such a length-determining element comes from the high degree of constancy in the distance from TATA to start (25-30 base pairs (bp)) in genes of higher eukaryotes⁵. It is less clear whether an equivalent element exists in *S. cerevisiae*, as the distance is quite variable, in the approximate range 35-180 nucleotides in the genes examined to date⁶.

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Electrostatic orientation during electron transfer between flavodoxin and cytochrome *c*

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Various studies have shown that reaction rates between reversibly binding electron transfer proteins depend strongly on solution ionic strength¹⁻⁷. These observations suggest that intermolecular electrostatic interactions are important in facilitating the formation of a productive reaction complex. A recently examined system involves the reduction of vertebrate cytochrome *c* by bacterial flavodoxin^{8,9}. Although this is a non-physiological reaction, it proceeds with rates typical for natural partners and is similarly inhibited at high ionic strengths. Here we describe computational studies which examine the role of electrostatics in the formation of a putative reaction complex between flavodoxin and cytochrome *c*. The results suggest that electrostatic interactions preorient the molecules before they make physical contact, facilitating the formation of an optimal reaction complex.

Figure 1a, b shows the $2kT$ electrostatic potential fields around tuna ferricytochrome *c*¹⁰ at two ionic strengths. The fields were computed from the crystallographic coordinates¹¹ using a modified Tanford-Kirkwood theory which scales the local dielectric constant of the charged groups according to their solvent-accessible surface areas¹²⁻¹⁴. As first inferred from comparative structural studies^{15,16}, and subsequently verified by Koppenol *et al.*¹⁷, the presence of a 'necklace' of positively charged lysine residues around the perimeter of the haem crevice leads to a pronounced asymmetry in the electrostatic field of the molecule. Figure 1c, d shows similar representations for *Clostridium* MP flavodoxin¹⁸. Here it is again apparent that the molecule possesses a highly asymmetric potential field, in this case due to a concentration of negatively charged amino acids in the vicinity of the flavin mononucleotide (FMN) prosthetic group. Therefore, it is clear that the complementary electrostatic fields on these proteins can mutually orient them to facilitate a direct approach between the flavin and haem prosthetic groups.

Figure 2 shows stereoscopic views of a putative reaction complex of flavodoxin and cytochrome *c* generated by computer graphics^{8,19,20}. This arrangement minimizes the prosthetic group separation subject to intermolecular steric constraints at the

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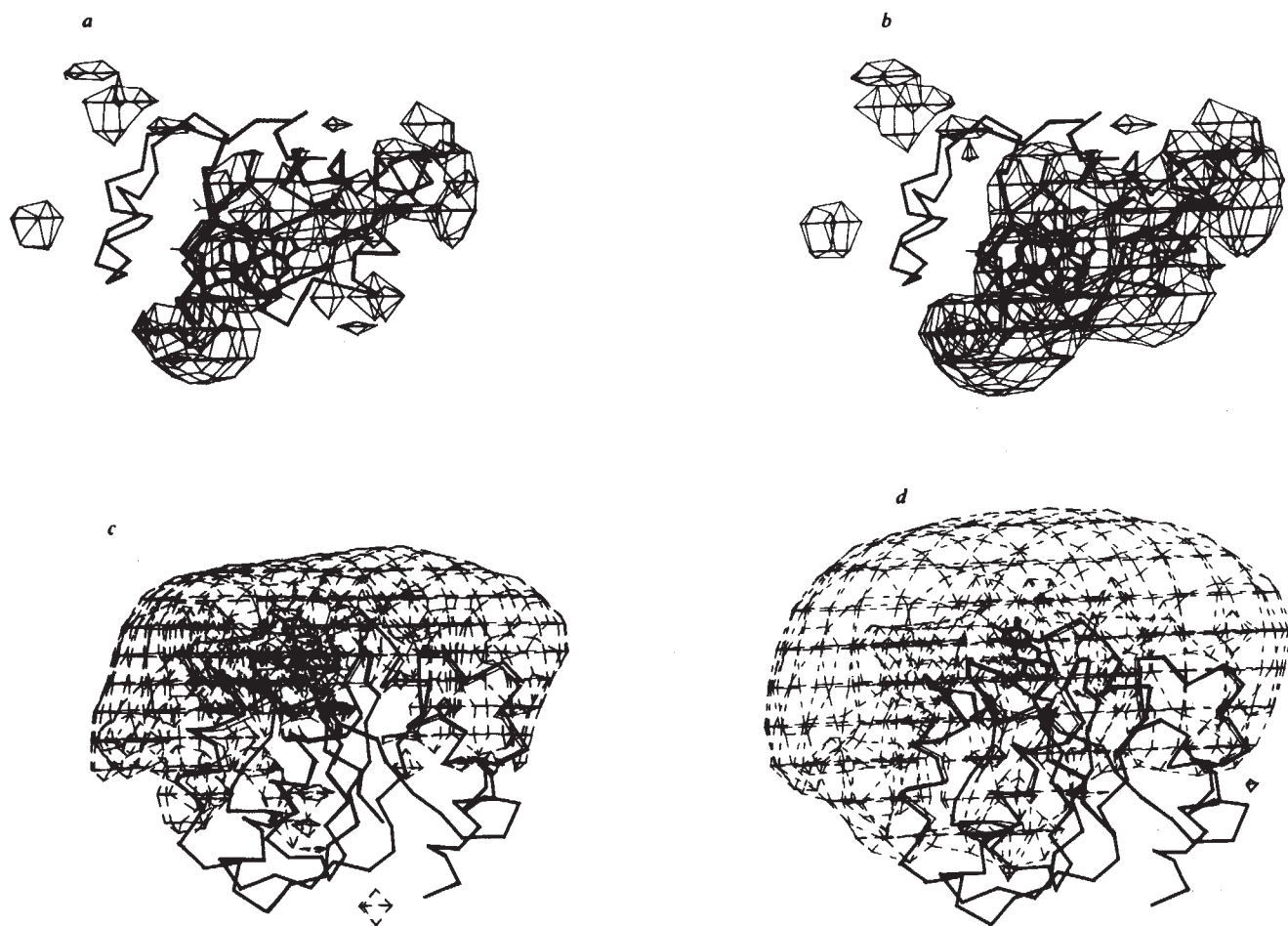


Fig. 1 Electrostatic fields, contoured at an energy level of + or $-2 kT$ ($=1.2 \text{ kcal mol}^{-1} = 0.052 \text{ eV}$) around tuna ferricytochrome *c* (a, b) and *Clostridium* MP flavodoxin (c, d) at pH 7 and solution ionic strengths of 0.08 M (a, c) and 0.04 M (b, d). The C^α chains of the two proteins are shown as heavy lines. Cytochrome *c* and flavodoxin have respective net charges of +7 and -18. The fields are asymmetrically distributed on the molecular surfaces near the prosthetic groups. The scale of these diagrams and those in Fig. 2 can be estimated from the C^α - C^α distance in the backbone chains which is about 4 Å.

interface in the complex, and additionally incorporates four specific ionic interactions between lysine residues on cytochrome *c* and acidic residues of flavodoxin⁸. (These are the same charged groups whose close proximity on the isolated molecules makes a predominant contribution to their local electrostatic fields^{8,19}.)

To assess the role of complementary electrostatics in the formation of the complex, we have computed the change in electrostatic free energy during both reactant association (ferricytochrome *c* and flavodoxin semiquinone) and product dissociation (ferrocyanochrome *c* and oxidized flavodoxin) assuming a simplest case trajectory where there is no relative rotational reorientation of the molecules. It has been suggested previously that the driving force for complementary electrostatic association reflects the participation of two factors: (1) the stabilization which occurs on complex formation because the groups of each molecule have like charge, and (2) the reduction of local dielectric constant at the molecular interface that results from solvent exclusion as the molecules associate^{16,19}. For molecules having asymmetric charge distributions, a complementary electrostatic complex is more stable than the isolated molecules. The interaction energy decreases smoothly on association and reaches a minimum when the reaction complex is formed (Fig. 3). The subsequent loss of a cytochrome *c* positive charge when it is reduced destabilizes the product complex by $\sim 1 \text{ kcal mol}^{-1}$, a relative difference between reactants and products which is preserved as the molecules dissociate. The scaling factor for each charged atom, which is

proportional to its solvent-accessible surface area^{12,15}, can be viewed as a quantitative measure of the extent to which the water (or ionic solvent) surrounding each group shields its charge¹⁴. We have estimated the extent to which solvent exclusion, or equivalently, the drop in local dielectric constant, enhances the electrostatic free energy of association. The association energy profiles were computed with the accessible areas left at their infinite separation values (dashed curve of Fig. 3) or with the areas recomputed at each separation distance to allow for solvent exclusion (solid curve of Fig. 3). The difference between these curves suggests that solvent exclusion enhances the electrostatic interactions by an amount corresponding to about one-half of the total stabilization energy.

In Fig. 4, observed apparent and computed association constants⁸ are plotted as a function of ionic strength. The close correspondence suggests that electrostatic interactions make the principal contributions to the free energy of complex association, and that entropic effects due to solvent release essentially compensate for those arising from reduction in independent molecular degrees of freedom²¹⁻²³.

The role of electrostatic interactions in enhancing the kinetics of the flavodoxin-cytochrome *c* reaction can be summarized in terms of two interrelated effects. First, the interaction of the electrostatic fields preorients the molecules along a trajectory leading to close approach of the prosthetic groups^{7,15,16,19,20}. Thus, instead of random collisions, the fields interact preferentially so that only a single degree of rotational freedom (along an axis connecting prosthetic groups) remains to be defined in

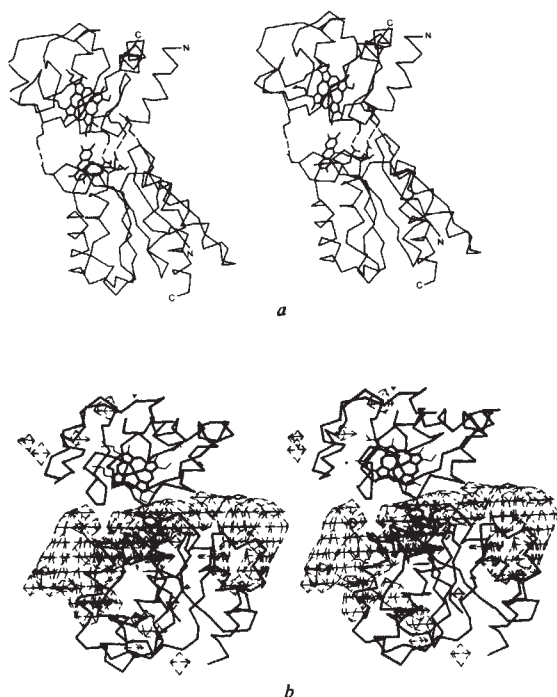


Fig. 2 Stereoscopic views of a computer-generated complex of *Clostridium* MP flavodoxin and tuna cytochrome *c*. In *a*, the C^α chains only are shown. Side-chain interactions constrain the relative orientations of the haem and FMN prosthetic groups and exclude solvent from the molecular interface. Four complementary ionic interactions (dotted lines) were optimized by slight reorientations of the crystallographically observed conformations of interacting charged residues. In *b* the complex is seen from a different aspect, together with its $2 kT$ electrostatic potential field at pH 7.0 and 0.08 M ionic strength. The field is wholly negative and reflects discharge of the positive field of cytochrome *c* on association with the more highly charged flavodoxin molecule.

the formation of the structurally unique complex. This orientation effect becomes significant when the interaction free energy between the molecules is about $2 kT$ less than the sum of the free energies of the isolated states. In the physiological conditions simulated in Fig. 3, this occurs when the molecules are still physically separated by about $6.5 \text{ \AA}$. Second, the fields could serve additionally to accelerate the actual association during complex formation^{7,15,16,19,20}. This effect may account for the observed increase in flavodoxin–cytochrome *c* association rates⁸ at decreased ionic strengths (Fig. 4). Figure 1 shows that a decrease in solution ionic strength deshields the molecules so that they undergo attractive interactions at larger separation distances.

Some of the general properties considered here may apply to other biological systems where complementary electrostatic interactions are important, for example protein–DNA and protein–membrane interactions. It might be anticipated that field interactions similar to those described above could constrain protein diffusion on DNA²³ or membrane surfaces^{16,25} to one or two dimensions, respectively. Interestingly, the present results (Fig. 3) suggest that when a proper (that is solvent-excluding) fit is achieved between the protein and a complementary structural feature of the DNA or membrane substrate, there is a dramatic increase in the binding free energy. The electrostatic forces can consequently act in concert with other specific steric or hydrogen-bonding interactions to immobilize the protein in a specific complex.

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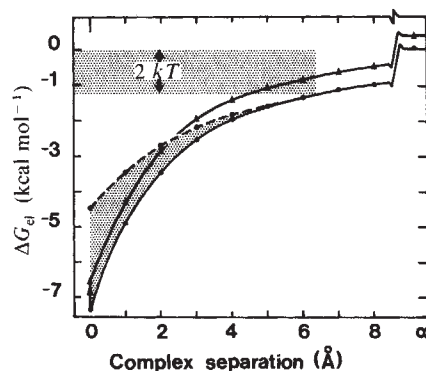


Fig. 3 The calculated change in electrostatic free energy (ΔG_{el}) on association of ferricytochrome *c* and flavodoxin to form the complex shown in Fig. 2 (pH 7.0, $I = 0.08 \text{ M}$) is indicated by the dots and the solid curve, and by the triangles, the dissociation behaviour after cytochrome *c* reduction. The difference between the dashed and solid association curves (shaded) reflects the enhancement of ΔG_{el} due to solvent exclusion as the complex is formed. The molecules begin to orient when ΔG_{el} becomes more favourable relative to the isolated molecules by $2 kT$, that is at $\sim 6.5 \text{ \AA}$ separation.

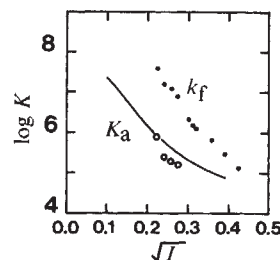


Fig. 4 The variation in the computed association constants ($\log K_a = -\Delta G_{el}/2.3RT$ from Fig. 3) for the complex as a function of solution ionic strength is shown by the solid line. $\circ$, Observed values of K_a (apparent) derived from kinetic studies on electron transfer rates between cytochrome *c* and *Clostridium pasteurianum* flavodoxin semiquinone, a molecule homologous to the *Clostridium* MP species used for the computational studies. $\bullet$, Observed ionic strength dependence of the association rate constants for the latter system (referred to the same ordinate log scale). The observed rate accelerations may reflect progressively longer-range electrostatic orientation effects as the ionic strength is decreased (see Figs 1, 3).

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Covalently closed circles of adenovirus 5 DNA

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The genome of adenoviruses is a double-stranded linear DNA molecule with inverted terminal repeats about 100 base pairs (bp) in length^{1,2} and a terminal protein covalently linked to the 5' nucleotide of each strand^{3,4}. Both of these features permit the formation of DNA circles, the inverted repeats allowing the circularization of single-stranded DNA^{5,6} and the terminal protein the joining of one or more molecules to yield double-stranded circles or concatemers⁷. However, although the existence of covalently closed circles has been postulated⁷, double-stranded viral DNA purified from virions or infected cells by conventional methods (that is, using proteases and phenol or chloroform) has always been obtained in a linear form⁸. Here we present evidence for the existence in adenovirus 5 (Ad5) infected cells of novel structures resulting from covalent head-to-tail joining of viral DNA molecules and show that these structures are due at least in part to the formation of covalently closed circles.

Several studies have shown that only the left 10–12% of adenovirus 2 (Ad2) or Ad5 DNA is required for transformation of rodent cells, and transformed cells usually contain only viral DNA sequences from the left end^{9–11}. However, occasionally a larger fraction of the viral genome is present in transformed cells and in certain conditions of infection virtually the entire viral DNA molecule can be integrated co-linearly into the host chromosome^{12–14}. In addition, several examples exist of transformed cell lines in which the left and right ends of viral DNA are linked together^{15,16}. These observations suggested involvement of both ends of the viral genome in the integration process, possibly through the formation of circular intermediates, and prompted us to examine the structure of intracellular viral DNA sequences at early times during transformation. Our initial experiments were carried out with host range mutant hr 1 (ref. 17) because we had previously found that this and other hr mutants of group I induced transformation of BRK cells at abnormally high efficiency¹⁸, and the transformants frequently seemed to contain the entire viral genome co-linearly integrated¹⁴. Southern blot hybridizations (Fig. 1) showed that restricted DNA from infected cells contained, in addition to the expected free terminal fragments, a novel fragment which migrated more slowly and hybridized to both left end (Fig. 1a) and right end (Fig. 1b) probes. For all three restriction enzymes, the size of this novel fragment, calculated using the partial *Hind*III digest in lanes 2 as a molecular marker, corresponded to that predicted for a fragment resulting from head-to-tail joining of the viral genome.

Head-to-tail joining of Ad5 DNA was not unique to hr-1 infected BRK cells, although it was most easily detected in cells infected with group I hr mutants (hr 3 and hr 5 behave similarly to hr 1). In subsequent experiments we detected joint fragments in wt (Fig. 2a) or group II mutant (Fig. 2b, lane 3) infected BRK cells, although only at later times (at least 72 h) after infection. In addition, head-to-tail joining occurred in established rat cell lines (3Y1 and 1074–9, Fig. 2b, lanes 5, 6) as well as in HeLa cells (Table 1). Notably, head-to-tail joining was never detected in DNA extracted from purified virions whether of hr 1 or wt origin. The results of several experiments with hr 1 infected BRK cells, summarized in Table 1, indicated that joint structures could be detected in small amounts as early as 3 h after infection and were present in relatively constant amounts (8–15%) from 5 to 120 h after infection. Comparable results were obtained with HeLa cells.

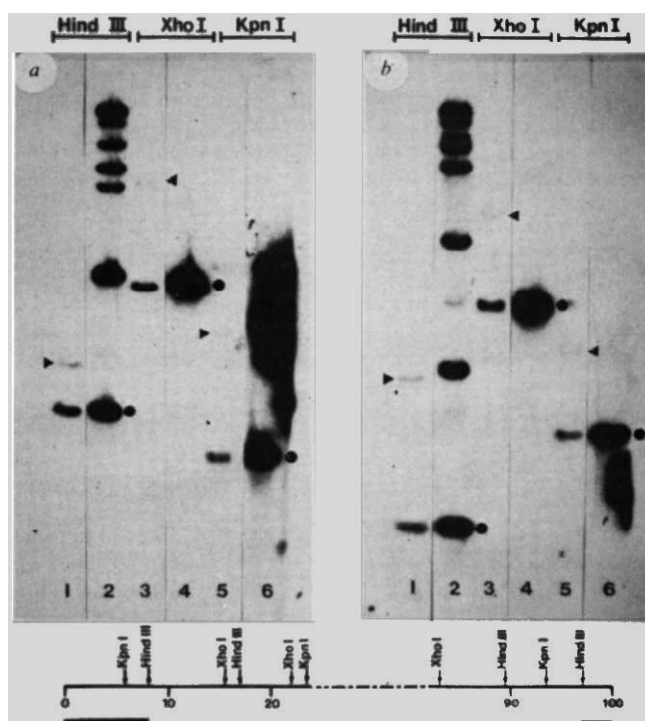


Fig. 1 Detection of head-to-tail joining of Ad5 DNA molecules in primary BRK cells infected with hr 1 mutant virus. *a*, The results of hybridizations with the *Hind*III G left-end terminus of Ad5 DNA; *b*, results obtained with the *Hind*III I right-end terminus.

Methods: Subconfluent cell monolayers were infected with hr 1 (group I) mutant Ad5 at a multiplicity of 1 plaque forming unit (PFU) per cell. Eight hours after infection, the monolayers were rinsed with phosphate-buffered saline, incubated overnight at 37 °C in 10 mM Tris pH 7.5, 10 mM NaCl, 1 mM EDTA, 0.4% SDS, 0.5 mg ml⁻¹ pronase, after which nucleic acids were extracted with Tris buffer-saturated phenol and chloroform isoamylalcohol (24:1)¹⁴. Following treatment with RNase A (10 µg ml⁻¹ for 30 min at 37 °C), re-extraction with chloroform isoamyl alcohol and dialysis against Tris buffer, the DNA was precipitated with ethanol and resuspended in 20 mM Tris pH 7.5, 1 mM EDTA. 10 µg of each DNA preparation were digested with restriction endonucleases *Hind*III, *Xho*I or *Kpn*I and electrophoresed on 0.7% agarose gels. The DNA was denatured *in situ* and transferred to nitrocellulose filters according to the procedure of Southern³² as modified by Ketner and Kelly³³. DNA probes, consisting of plasmids carrying the *Hind*III G (0–8 mp) or *Hind*III I (97–100 mp) terminal fragments of Ad5 DNA³⁴ were labelled *in vitro* with [α -³²P]dCTP according to Maniatis *et al.*³⁵. Hybridization was carried out according to Wahl and Stark³⁶ and filters were exposed to Kodak X-ray films XR5 at –70 °C with intensifying screens. In each panel, odd-numbered lanes contain DNA extracted from infected cells and even-numbered lanes contain marker Ad5 DNA mixed with DNA from uninfected BRK cells before restriction. The *Hind*III digest in lanes 2 is a partial digest which serves as size marker. The restriction map of Ad5 DNA at the bottom of the figure shows the relevant restriction endonuclease sites at the left and right ends of the genome. The map location of the *Hind*III G and I fragments used as probes is indicated by solid bars. Free viral DNA termini in the infected cell DNA are identified by closed circles. Joint fragments, discussed in the text, are indicated by arrows. The hybridization band above the joint fragment in *a*, lane 5, results from hybridization between *Hind*III G (0–8 mp) and *Kpn*I A (5.8–23.5 mp).

Head-to-tail joining of Ad5 DNA molecules could result from circularization or from formation of concatemers. To detect covalently closed circles of Ad5 DNA, primary BRK cells infected with hr 1 and labelled with ³H-thymidine were lysed in alkali, and denatured DNA and protein were precipitated by neutralization with acetate buffer. The supernatant from this extraction (presumed to contain renatured covalently

Fig. 2 Detection of head-to-tail joining of Ad5 DNA molecules in primary BRK or in established rat cell lines infected with wt or hr mutant Ad5. The results shown were obtained with DNA digested with *Hind*III (a) or *Kpn*I (b) and hybridized with the *Hind*III G fragment of Ad5 DNA.

Methods: For details of infection, DNA extraction and digestion, electrophoresis and hybridization see Fig. 1 legend. a Illustrates the time course of appearance of joint fragments (▶) in BRK cells infected with hr 1 (lanes 1, 3, 5) or wt Ad5 (lanes 2, 4, 6). Total DNA was extracted from the cells at 8, 20 and 72 h, respectively. Lane 7 contains a partial *Hind*III digest of Ad5 DNA as a molecular weight marker. b Shows the presence of joint fragments (▶) in BRK cells infected with hr 1 (group I), hr 6 (group II) and wt Ad5 (lanes 2, 3, 4, respectively) and collected 120 h after infection. Lanes 5 and 6 refer to DNA extracted from established rat cell lines (3Y1³⁷ and 1074-9 respectively (F.L.G. unpublished)) 72 h after infection with hr 1. Marker Ad5 DNA (complete *Kpn*I digest) is shown in lane 1. In all lanes the most slowly migrating band results from the hybridization of the *Hind*III G probe to the *Kpn*I A fragment of Ad5 DNA, as discussed in Fig. 1 legend.

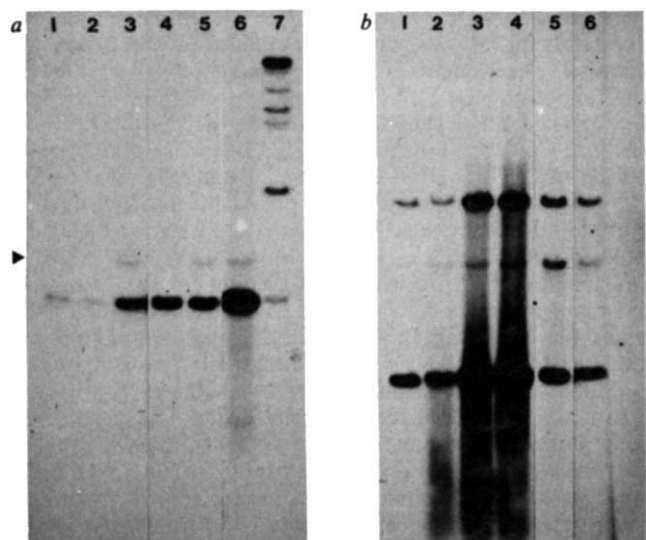
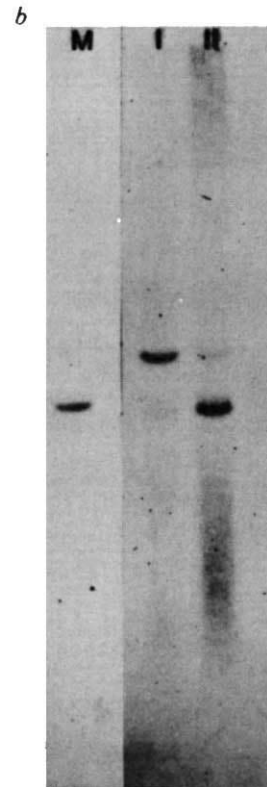
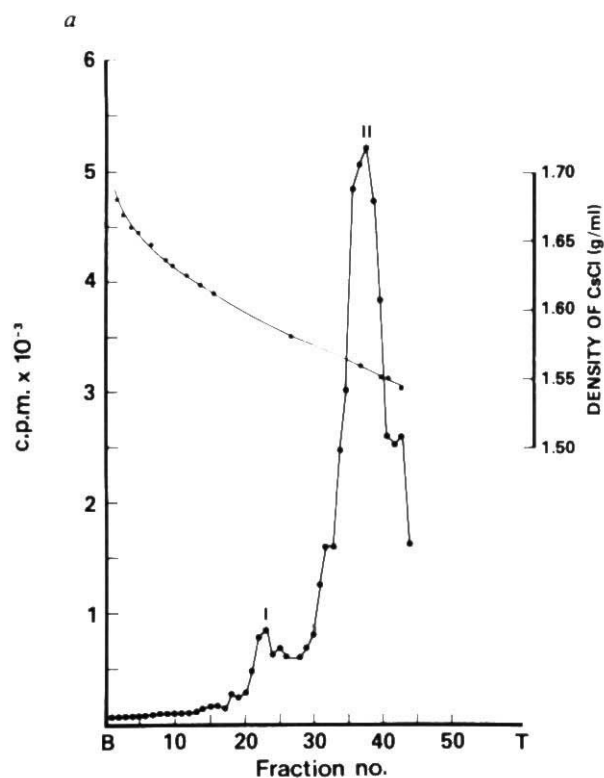


Fig. 3 Characterization of Ad5 DNA from infected BRK cells by equilibrium sedimentation. a, Size fractionation on CsCl-ethidium bromide gradients; b, results of hybridization of DNA from peaks I and II using *Hind*III G fragment.

Methods: a, Primary BRK cells were infected with Ad5 hr 1 mutant at a multiplicity of 1 PFU per cell. ³H-thymidine (0.5 μ Ci ml^{-1}) was added at both 24 and 48 h after infection and the cultures were collected at 72 h. Covalently closed circular DNA was partially purified following a modification of the procedure developed by Birnboim and Doly³⁸ for purification of plasmid DNA from bacteria. Specifically, the cells were washed with phosphate-buffered saline and lysed for 10 min at room temperature by addition of alkaline SDS (2% SDS, 0.4 M NaOH in 50 mM glucose, 25 mM Tris, 10 mM EDTA) and 3 M sodium acetate pH 4.8 was added to a final concentration of 1 M for 1 h at 4 °C. Following centrifugation the DNA in the supernatant was precipitated with ethanol and resuspended in 20 mM Tris pH 7.5, 1 mM EDTA. CsCl and ethidium bromide were then added to a final density of 1.60 g cm^{-3} and a concentration of 200 $\mu\text{g ml}^{-1}$, respectively. The gradients were centrifuged to equilibrium at 35,000 r.p.m., 15 °C for 65 h in a Beckman 50 Ti rotor and were then fractionated by collecting 10-drop fractions from the bottom of the tube. Aliquots (20 μ l) from each fraction were counted in a liquid scintillation counter and the radioactivity profile shown was obtained. A second aliquot from most of the fractions was used to measure the refractive index from which the density along the gradient was calculated. Fractions corresponding to peak I (18–25) or peak II (28–40) were then pooled, extracted with isoamylalcohol to remove ethidium bromide and dialysed. b, DNA recovered from peaks I and II of the gradients was digested with *Hind*III, and analysed by Southern blot hybridization as described in Figs 1, 2 legends using *Hind*III G fragment as labelled probe. A complete *Hind*III digest of Ad5 DNA purified from virions was used as marker (M).



closed circles) was centrifuged on CsCl-ethidium bromide equilibrium gradients which were then fractionated. Counting of aliquots from each fraction revealed the presence of two distinct peaks of radioactivity (Fig. 3a), peak I banding at a density ($\sim 1.60 \text{ g cm}^{-3}$) typical of covalently closed circular DNA, and peak II at 1.56 g cm^{-3} . DNA from each of the peaks was digested with *Hind*III and analysed by Southern blot

hybridization using *Hind*III G as the probe. As can be seen from Fig. 3b, Ad5 DNA from peak I was almost exclusively in the form of joint fragments, with only trace amounts of label hybridizing to free *Hind*III G fragment. As in previous experiments, the joint fragment also hybridized to the *Hind*III I right-end probe (not shown). In contrast, peak II contained predominantly linear viral DNA, as most of the label hybridized

to a fragment co-migrating with the *Hind*III G fragment in the marker channel (M). We cannot calculate the proportion of head-to-tail joints due to circles because we have no estimate of the loss of circular molecules (due to formation of single-strand nicks or other causes) incurred during the purification procedure. However, the results of Fig. 3 establish that at least a portion of the hr 1 DNA in infected BRK cells must be in the form of covalently closed circles; we assume that such circles also exist in the other experimental conditions shown to result in head-to-tail joining.

The simplest structure which can account for the observed joint fragments would result from flush end joining of left and right termini. However, although bands corresponding to joint fragments are reasonably well defined, their size cannot be determined with a precision greater than 100–200 bp. Consequently, the fine structure of head-to-tail joints remains obscure. Attempts to clone the joint fragments (F.L.G., unpublished) have consistently resulted in plasmids containing Ad5 left and right sequences but with deletion of all or most of the terminal repeated sequences, probably because these sequences, when joined together, generate a structure (a 200-bp palindrome) thought to be lethal for plasmids¹⁹.

Table 1 Fraction of viral DNA joined head to tail in hr 1 infected cells

Time post-infection (h)	BRK	Cells	HeLa
3	0.01		ND
5	0.08		ND
8	0.15		0.08
20	0.11		0.09
72	0.13		0.04
120	0.09		ND

The amount of viral DNA present as head-to-tail joints was determined by scanning autoradiographs using a Joyce-Loebl scanning spectrophotometer and is expressed as the ratio of label hybridizing with the joint fragment to the sum of label hybridizing with the joint plus free terminal fragment. ND, not determined.

What is the significance of head-to-tail joining (and formation of circles) of Ad5 DNA in infected cells? Circles and concatemers of viral DNA could be precursors for DNA replication or be involved in integration. It is now well established that the terminal protein linked to adenovirus DNA is involved in viral DNA replication, possibly acting as a primer for initiation of DNA synthesis^{3,20–22}. However, none of the studies carried out to date eliminates the possibility that the terminal protein, or its precursor of molecular weight 80,000, is also a nicking-closing enzyme which could generate covalently closed circles, a possibility first suggested by Robinson *et al.*⁷, or that the terminal protein functions in DNA replication as an origin-specific topoisomerase²³. Alternatively, a cellular enzyme may be involved in covalent joining of viral DNA termini and the terminal protein might act to bring the ends together, a property it is known to possess^{3,7}. Detection of the joint fragment as early as 3–5 h post-infection suggests that incoming molecules may be circularized before the onset of viral DNA replication. However, absence of joint fragments in DNA extracted from purified virions suggests that covalently closed circular viral DNA is rarely if ever packaged as such, even though as much as 10–15% of the intracellular viral DNA is joined head to tail.

For several reasons it also seems probable that circles of Ad5 DNA may play a role in integration. First, conditions which most efficiently generate head-to-tail joining—infection of

BRK cells with group I hr mutants—also result in exceptionally high transformation frequencies¹⁸ and often give rise to transformants containing virtually the entire viral genome covalently integrated into cellular DNA¹⁴. In addition, linked terminal fragments have been detected in Ad2 and Ad5 transformed cell lines and it has been suggested that circularization may precede integration^{15,16}. Second, circular molecules as precursors of integration have been proposed for several retroviruses. Covalently closed circular (form I) proviral DNA apparently originates from linear double-stranded DNA molecules²⁴, and inhibition of synthesis of form I DNA also seems to block integration^{25–29}. Third, circular viral DNA molecules have been detected in Epstein-Barr virus transformed cells³⁰, and circular *copia* molecules have been detected in *Drosophila* cells³¹, additional examples of systems in which DNA sequences may be inserting in (or excising from) cellular DNA.

To elucidate the role, if any, of head-to-tail joining of adenovirus DNA molecules in DNA replication and transformation, it will be useful to examine the effect of agents which have been found to block circularization of retrovirus genomes. In addition, sequencing of Ad5 joint fragments as well as of the cell-virus joint in hr group I transformed cell lines might indicate whether the mechanism of adenovirus DNA insertion into host DNA shares any similarity with retrovirus DNA integration.

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Steady-state magma discharge at Etna 1971–81

ESTIMATIONS by Wadge and Guest¹ of the volumes of lava emitted by each individual eruption of Mount Etna between 1971 and 1981, should be viewed with caution^{2,3}. Despite the impressive number of references (see Table 1 in ref. 1), very few of them contain accurate determinations of the volumes of lava erupted, that is: measurements of the surface covered by each lava flow and a sufficient number of determinations of the thickness. Regarding this latter point, it is symptomatic that all but one (J. B. Murray) of the authors to whom reference is made fail to mention any thickness determination, so that the volumes quoted are in most cases highly speculative: they are not measurements in a scientific sense⁴.

Let us now examine specific points:

- 5 April–12 June 1971: since the error bar of the volume estimate of this flank eruption is unknown, there is no means of inferring that the accuracy is 'probably' better than 50%. For another flank eruption (1950–51), calculations by J. B. Murray (personal communication) from the 1932 and 1969 IGM maps lead to a value of $116 \times 10^6 \text{ m}^3$ ($\pm 10\%$), instead of $55 \times 10^6 \text{ m}^3$ as 'estimated' by Wadge².
- June–September 1971/October 1973: no lava effusion occurred during this period. It is difficult to imagine the meaning of the $17 \times 10^6 \text{ m}^3$ listed. If it represents the filling of the Central Crater Chasm, it must be pointed out that the dimensions of its interior were not known³.
- Eruptions from the NE Crater and the northern flank, 1974–76: except for the small area ($800 \times 500 \text{ m}$) around the NE Cone^{5,6} and for a small part of the lava field (very approximate sketch in ref. 7), there is no map of the erupted products. How, therefore, can their volume be calculated?
- Eruptions from the NE Crater, 1977–78: we have a succession of 17 eruptions, most of which occurred at intervals of a few days in a zone of the volcano which is almost inaccessible during the winter. Not only is there no available map, but the exact location of many flows is not even known. Similar observations can be made for the 1980–81 eruptions of the same NE Crater: the authors to whom reference is made¹ indicate only the approximate length of the lava flows.
- Flank eruptions, 1978–79: none of these flows has been mapped so far. The sketch shown by Mackey and Scott⁸ only represents a small part of the flow corresponding to the less important of the four eruptions.
- March 1981 flank eruption: this is the only case where a detailed map has been

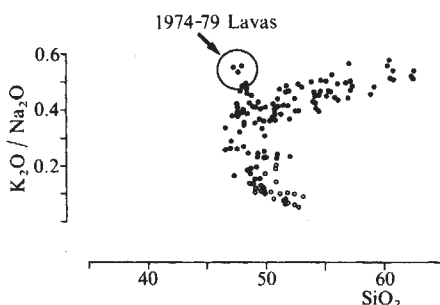


Fig. 1 Composition of the 1974–79 lavas compared with that of all the products of Mt Etna ($\text{K}_2\text{O}/\text{Na}_2\text{O}$ versus SiO_2). ○, Pre-1974 tholeiitic basalts; ●, alkaline lavas from the strato-volcano of Etna itself, including historic flows (from ref. 4).

made⁹ and a volume actually measured. It represents less than 10% of the total volume taken into account by Wadge and Guest¹ during the period 1971–81.

Therefore, I consider the conclusions drawn by Wadge and Guest to be unfounded, unless they can display a precise topographic map for each of the 35 eruptions listed in their Table 1. Although a steady-state magma discharge appears obvious to many observers for some periods of Mount Etna's 'persistent activity', it cannot be quantified with such speculative estimates. The observations by Italian, British and French researchers only permit the definition of an order of magnitude and we must await extensive topographic work to know with a sufficient degree of accuracy the volume of lava erupted in the decade 1971–81.

Finally, it is incorrect to emphasize the chemical homogeneity of recent lavas of Etna. Since 1974, a significant change in the composition of the products has occurred, with an evolution towards a more potassic character (see Fig. 1)⁴. Added to the exceptional frequency of the eruptions in recent times, this observation seems to indicate a new magma supply from a deep source and does not allow us to guess the future behaviour of Mount Etna.

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WADGE AND GUEST REPLY—Tanguy argues that our interpretation of magma discharge at Etna during 1971–81 is invalid because our estimates of lava volumes are too imprecise. His principal argument is that detailed maps and measured lava thicknesses are not available in most of the references quoted by us. We would point out, however, that our volume estimates are not based on information from these references alone, and that one of us has visited the volcano on average twice a year during the past decade and wherever possible has mapped the areal extent of new lavas and made thickness measurements. These data were used in our calculation of lava volumes and indeed are in part the basis of our study.

Thus, although Tanguy states that the 1981 eruption is the only one where detailed mapping and thickness measurements were made (by one of us and other UK workers), similar measurements were made on flows from other major eruptions. Where we were not able to obtain such data, especially for short-lived NE Crater eruptions, we have relied on eyewitness reports of others, for example R. Romano (personal communication) for the July 1977 eruption and C. R. J. Kilburn (personal communication) for the September 1980 eruption. The eruptions mentioned for June and September 1971 and October 1973 were indeed eruptions on the floor of the Chasm and the Bocca Nuova. Both these eruptions occurred when the internal dimensions of these two pits in the central crater were known from visual observations and range-finder measurements. Our original letter was an inappropriate place to justify each estimated volume.

Tanguy appears to accept our model of a steady-state magma discharge but rejects our volumetric data as 'speculative estimates'. However, he does not present any new data that demonstrate our estimates to be less precise than stated in our letter, and indeed the only quantitative data given by him are for an eruption that occurred in 1950–51 which is outside the period of our study.

With regard to Tanguy's comments on lava chemistry, each of the erupted lavas was hawaiitic in common with most historical lavas; but as we pointed out, there were limited variations in composition. Some of these occurred within single eruptions including those of 1971¹ and 1981 (S. Scott, personal communication) when chemically zoned magma-filled fissures may have developed during short repose periods before eruption. Other small variations in chemistry between one set of eruptions and another have been observed during the last decade and may identify individual batches of magma with different chemical signatures (S. Scott,

personal communication). The careful monitoring of Etna's output is clearly important in interpreting these chemical observations in terms of the volcano's internal plumbing system.

With regard to the prediction of an eruption in May 1982 ± 3 months, the intermittent presence of lava at the bottom of the Bocca Nuova (20 May–3 June, 9–20 August, 25–30 (?) September, observations by G. Patanè, Istituto di Scienze della Terra di Catania, and by members of the PIRPSEV teams) must be related to the normal oscillatory motion of the magma column. This activity has not brought any appreciable amount of new material as yet (5 December 1982). Similar activities occurred in 1981 (mid-May, mid-July, early September) and during summer 1980, including the SE Crater, (refs 2, 3): they were not interpreted as 'eruptions' by scientists and people concerned with Mount Etna. (At any rate, the prediction of an eruption with ± 3 months uncertainty is not significant for a volcano which erupts almost each year and often several times a year. Even had there been an eruption within this 6 months interval, it could have been a mere coincidence.)

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Identity of different mutations for deleterious genes

IN an analysis of β -thalassaemia in Mediterranean populations, Orkin *et al.*¹ have suggested that only eight separate mutational events leading to gene dysfunction are represented in 91 chromosomes taken from β -thalassaemic patients. This is perhaps the first time that such a statement on individual mutations has been possible on samples from human populations. They show further that there is a close association between mutational events and the pattern of restriction enzyme cleavage sites in the neighbourhood of the β -globin gene cluster.

We present here a theoretical treatment of this problem of identity of deleterious mutations in the framework of the classical 'infinite alleles' model of variation in finite populations, due to Kimura and Crow². They assume that at the locus concerned each mutation is to a novel type and that all genotypes have equal fitness. If u is the total mutation rate per generation and N is the effective population size, they show that at equilibrium the probability that two genes chosen at random are identical by descent is $1/(4Nu + 1)$. This expression can be simply derived by an equation in which

the reduction in identity by new mutation is balanced against the increase due to finite population size. If f_t is the probability that a randomly chosen pair of genes in generation t are identical by descent, we may write

$$f_{t+1} = (1-u)^2(1/2N + (1-1/2N)f_t) \quad (1)$$

where $(1-u)^2$ is the probability that neither is a new mutation and $1/2N$ is the probability that they both derive from the same gene in generation t . Putting $f_{t+1} = f_t$ leads to $f = 1/(4Nu + 1)$.

Consider a group of recessive lethal mutations at a locus which do not complement one another and which have equal fitness in heterozygotes with wild-type alleles. If u is the total mutation rate to such alleles and q is their total frequency, then the probability that neither gene of a pair is a new mutation is $(q/(q+u))^2 \approx (1-u/q)^2$. The number of mutant alleles in the population is $2Nq$, so that the expression $1/2N$ in (1) must be replaced by $1/2Nq$. Thus the probability of identity at equilibrium is again $1/(4Nu + 1)$, with u now the mutation rate to lethal alleles. Note that this is independent of any selection on the heterozygote over wild type (provided this is the same for all alleles) as q can be taken as the frequency of mutant alleles after selection. The approach does in fact apply to any homogeneous subset of mutants at a locus.

The probability of identity will be increased by non-random mating, for example in local populations with limited migration, and in an expanding population it will be higher than current population sizes would suggest. The probability is also changed if not all deleterious homozygous types are equally unfit or there is any complementation of different mutant alleles.

For lethal genes, the expected value of q^2 equals the total mutation rate, u , giving $f = 1/(4Nq^2 + 1)$ or $f = 1/(4L + 1)$, where L is the total number of affected individuals (called clinical homozygotes by Orkin *et al.*¹) in the population. The expected number of affected individuals which are homozygous for the same mutation is

$$Lf = L/(4L + 1) = Nu/(4Nu + 1)$$

which is less than one-quarter for all Nu . Thus, with lethality, the number of identical homozygotes is expected to be very small. If there is some heterozygote superiority for the mutant types, the number of affected individuals exceeds Nu and the number of identical individuals may exceed one-quarter.

Over a 60-kilobase length spanning the β -globin gene cluster, Orkin *et al.*¹ found in their sample of 91 chromosomes carrying β -thalassaemia mutations only nine patterns of presence or absence at a set of seven polymorphic restriction enzyme recognition sites, which they called 'chromosomal haplotypes'. The most

frequent haplotype made up 47% of the total. By sequencing the DNA of the β -thalassaemia genes on a number of chromosomes, they found only eight different types of mutations and, further, there was almost a one-to-one association of haplotype and thalassaemia mutation. As the frequency of haplotypes is similar in a sample of chromosomes with normal β -globin genes³, this implies that the thalassaemic mutations have occurred in a background of previously existing haplotype variation.

If this is so, we believe that they may have underestimated the number of thalassaemic mutational events by their experimental strategy as they deliberately concentrated their sequencing work on new haplotypes and only sequenced four samples of the most frequent haplotype (their Table 3): "Repeated isolation of the same gene was largely avoided by selecting β -genes for sequencing on the basis of their associated haplotypes". The actual probability of identity of mutants is likely to be lower than the value of 0.26, the observed identity of the restriction haplotypes. It is surprising that the frequency of haplotypes should be similar in normal chromosomes and in those carrying thalassaemia mutants and yet the more frequent haplotypes do not have proportionally more types of mutation.

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ORKIN *ET AL.* REPLY—Robertson and Hill show mathematically that if there is an infinite number of lethal, non-complementary alleles at a locus such as β -globin, then the expected number of identical homozygotes in the population is less than 0.25. Thus, in the presence of random genetic drift, irrespective of the population size one will rarely find identical homozygotes and only occasionally more than one. In other words, under strict lethality nearly all affected individuals will be genetic compounds. This, of course, will not be true if there is some inbreeding in the population.

These authors argue that sequencing of β^{thal} alleles from the same haplotype will yield new and different β^{thal} alleles. In Orkin *et al.* haplotype I accounts for about 50% of the haplotypes associated with β^{thal} in Mediterraneans. Robertson and Hill suggest that this haplotype, because it is frequent, might be expected to carry several different β^{thal} alleles. We now have definitive information regarding β^{thal} alleles in 13 of these haplotype I chromosomes. Four β^{thal} genes from this haplotype were sequenced and all had the

IVS-1 mutation described by Spritz *et al.*^{1,2} Nine others were studied by S₁ mapping of the erythroid RNA produced, and 8 out of 9 also had the IVS-1 mutation (T-c. Cheng, personal communication). Thus, 12 out of 13 haplotype I chromosomes studied in Mediterraneans had the same mutation. Presumably the majority of individuals homozygous for haplotype I are homozygous for this IVS-1 mutation.

We are therefore led to believe that our sequencing strategy for β^{thal} alleles is a valid one, because β^{thal} alleles are probably under positive selection in $\beta^A\beta^{\text{thal}}$ heterozygotes causing individual β^{thal} alleles to expand in the population. In other recessive genetic diseases in which positive selection in heterozygotes does not occur, for example galactosaemia, almost all patients may be genetic compounds and our sequencing strategy may not apply.

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Carbon isotope ratios of bone apatite and animal diet reconstruction

OUR model using $\delta^{13}\text{C}$ analyses of bone apatite to obtain dietary information from modern and fossil bones¹ has been disputed by Schoeninger and DeNiro who say that carbon isotope ratios in fossil bone apatite cannot be used to obtain dietary information because of postmortem exchange of carbon². We now discuss the evidence.

Although the details of analytical techniques reported by Schoeninger and DeNiro differ from ours, our only significant reservation is their use of 8.5M

acetic acid (50:50, v/v) for removal of normal carbonates. We have observed that glacial acetic acid has no appreciable effect on calcite and we wonder if the activity of 8.5M acetic acid is adequate to remove all normal carbonates, a critical pretreatment procedure. We use 1M acetic acid on the whole bone, and again after crushing to <1 mm powder.

The crux of their argument for postmortem exchange of carbon involves evidence from experiments on the ^{14}C dating of bone (refs 10–13 in ref. 2). Their ref. 10 contains no ^{14}C data, but the pertinent information is available elsewhere³. The appropriate ^{14}C data from their references 11 and 12, as well as the data from Hassan³ is presented in Table 1. ^{14}C activities relative to NBS oxalic acid are tabulated for pretreated apatite, for secondary carbonate, and for the age of the sample as it can best be evaluated by dating other materials. In addition, $\delta^{13}\text{C}$ analyses of bone apatite and of secondary carbonates are listed where available. Ref. 13 in ref. 2 did not use either collagen or apatite fractions and presents only data on samples which are likely to be contaminated, and thus is not considered here.

Most bone samples of archaeological interest are found in relatively near surface unconsolidated materials and are subjected to contamination with modern carbonate from downward percolating waters (relative ^{14}C activity 1.000). Such a situation is confirmed by all six examples in Table 1 where ^{14}C activities of secondary carbonates were measured. Using a ^{14}C activity of 1.000 for the exchanging carbonate, and a ^{14}C activity appropriate for the independently accepted true age of each sample, we can easily calculate the maximum amount of carbonate exchange that might have occurred for each of the analyses in Table 1. The largest possible exchange indicated is 6.9%, and the average indicated exchange is 3.0%. Whether this is actual isotopic exchange with bone apatite, or simply imperfect removal of modern secondary carbonates, cannot be demonstrated from these data. It is clear, however, that isotopic exchange of carbonate with bone apatite is limited to a few per cent.

Having demonstrated that exchange of

carbonate with bone apatite is very limited, let us examine the effect of the maximum indicated exchange on the isotopic data ($\delta^{13}\text{C}$) used for dietary information. In the references cited by Schoeninger and DeNiro there are four bone samples for which $\delta^{13}\text{C}$ analyses on both apatite and normal carbonates are reported (See Table 1). The carbonate $\delta^{13}\text{C}$ analyses differ from the apatite $\delta^{13}\text{C}$ analyses by 0.2–4.8%. A 3% exchange of carbon would lead to an error in $\delta^{13}\text{C}$ analysis of an apatite amounting to only 0.00–0.15%. This is less than the probable analytical error of the apatite $\delta^{13}\text{C}$ analysis itself. Such an error cannot significantly affect dietary interpretations using bone apatite $\delta^{13}\text{C}$ analyses.

Having shown that postmortem exchange does not seriously affect $\delta^{13}\text{C}$ analyses of bone apatite, we must still comment on the actual data presented by Schoeninger and DeNiro². Our paper¹ was based on data from 26 samples, 24 of herbivores and 2 of humans. Subsequent research in our laboratories has indicated that the model we put forward is applicable only to herbivores. Both carnivores and omnivores (including humans) require different models because of the complexity of their diets. Models are being developed which will readily explain most of Schoeninger and DeNiro analyses (18 of their 24 fossil samples are carnivores or omnivores). Our interpretation of bone apatite $\delta^{13}\text{C}$ analyses¹ appears to give appropriate dietary information for herbivores as old as 2 Myr (ref. 4). We continue to anticipate that $\delta^{13}\text{C}$ analyses on bone apatite from fossil bones will make valuable contributions to Quaternary and Pleistocene research.

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Table 1 ^{14}C and ^{13}C data from Schoeninger and DeNiro citations

Sample name and data source	^{14}C Activity of apatite	Expected ^{14}C activity*†	^{14}C Activity of carbonate*‡	Maximum carbon exchange (%)	$\delta^{13}\text{C}$ apatite	$\delta^{13}\text{C}$ carbonate‡
Folsom bison ³	0.322	0.272	1.068	6.9	−6.8	−6.4
Blackwater Draw mammoth ³	0.280	0.249	1.216	4.1	+1.8	−3.0
Domebo mammoth ³	0.277	0.255	NA§	3.0	−4.9	−7.0
Trolinger Bog mastodon ³	0.060	0.061	0.915	−0.1	−11.4	−7.1
Hudson Meng bison (ref. 11 in ref. 2)	0.327	0.295	1.153	4.5	NA§	NA§
Lehner mammoth (ref. 12 in ref. 2)	0.289	0.246	0.862	5.7	NA§	NA§
Hell Gap bison (ref. 12 in ref. 2)	0.324	0.343	0.917	−2.9	NA§	NA§

* ^{14}C activities are relative to modern (1950) carbon. † Expected activity is based on best independent estimates of age. ‡ Carbonate is the secondary carbonate usually evolved with acetic acid. § Not analysed or analyses not available.

SCHOENINGER AND DENIRO REPLY—Sullivan and Krueger raise three points that require discussion. First, they indicate that they have reservations about the method we used to remove normal (non-apatite) carbonates from bone. As we indicated¹, X-ray diffraction analysis demonstrated that calcium carbonate present in fossil bones was removed by our method.

Second, Sullivan and Krueger present ¹⁴C data from which they conclude that 'isotopic exchange of carbonate with bone apatite is limited to a few per cent'. This conclusion is based on calculations which depend on the assumption that fossil bone apatite undergoes exchange only with modern carbonate (carbonate with a ¹⁴C activity of 1.000) in groundwater. This assumption is not reasonable. After an animal dies and its bones are buried, the bone apatite could begin to exchange immediately with contemporaneous groundwater carbonate. The exchange process could continue up until the time the bone was excavated or might stop sometime earlier, due to changes in the depositional environment (for example, inundation of the sediment with oil) or in the accessibility of the apatite to groundwater (for example, sealing off the bone with secondary calcium carbonate or other mineral deposits). The ¹⁴C activity of the groundwater carbonate with which fossil bone apatite exchanged could range from 0 (for old bones in which the exchange process stopped long before excavation) to 1.000 (for bones in which the exchange process continued until excavation). This value cannot be estimated from the ¹⁴C activity of secondary carbonates deposited on the bones because these deposits may be contaminants of recent origin². Thus the amount of carbon isotope exchange that has occurred in fossil bone apatite cannot be calculated according to the method Sullivan and Krueger have used.

Finally, Sullivan and Krueger now indicate that their original model³ is applicable only to herbivores. We cannot comment on the models they are developing to explain the isotopic relationships between bone collagen and apatite for carnivores and omnivores. However, we cannot conceive of any model that could explain our observation¹ of an 8.3% range in the $\delta^{13}\text{C}$ values of bone apatite from eight individuals who lived during the Venta Salada phase of the Tehuacan Valley occupation, for whom the archaeological as well as the bone collagen carbon and nitrogen isotopic evidence all indicate the same diet.

We therefore stand by our original conclusion¹ that the ¹³C/¹²C ratios of fossil bone apatite cannot be used for dietary reconstruction until a method is developed to identify those specimens in which the apatite has not been subjected to postmortem exchange. One critical test of such a method would be its application

to bones from a group of individuals of a single species for which the archaeological or palaeontological data indicate the same diet. The ¹³C/¹²C ratios of diet reconstructed from the bone apatite carbon isotope ratios of such a group should have a small range and agree with the diet ¹³C/¹²C ratios as reconstructed from the bone collagen isotopic composition.

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Mycoplasma pneumoniae and a dual aetiology for Spanish oil syndrome

RECENTLY, Pestaña and Muñoz suggested¹ that Spanish oil syndrome was caused by a cytotoxic reaction to anilides based on a free radical pathology. The symptoms of this disease do not, however, correspond^{2,3} to toxicity due to aniline or to any of the other known components of the oil (for example, erucic acid, quinoline, toluene, benzene, glucosinates and short-chain hydrocarbons). No laboratory has found clinical toxicity due to aniline at levels comparable to that found in the oil^{1,4–7}.

Pestaña and Muñoz have suggested that the anilides are somehow "activated by free radicals in the oil". However, García-Gancedo *et al.*¹ found that vitamin E, a free radical scavenger, is ineffective in clinical trials: "there are no clinical data in support of free radical pathology". Thus, the available data support neither anilides as the prime toxic substance nor a free radical pathology. Indeed, denatured oils have been marketed in Spain for years without known adverse effects. Pestaña and Muñoz, without any data, assert that earlier oils must therefore have been "devoid of anilines". Other researchers alternatively conclude⁸ that "the incriminated oil is not *per se* the cause of the epidemic. Rather (the cause) is a combination of factors, some of which remain to be explained".

We suggest that one key factor has been overlooked. Spanish oil syndrome was first diagnosed as a form of atypical pneumonia. The Ministerio de Sanidad y Consumo published figures showing that *Mycoplasma pneumoniae* or other *Mycoplasma* were isolated from one-third of the cases studied¹⁰. Recent studies of *M. pneumoniae* indicate that it is very difficult to isolate and can only be diagnosed with certainty using antibody tests¹¹, and only

1 in 30 cases of *M. pneumoniae* infection are manifested as pneumonia⁹. Thus, the actual rate of *M. pneumoniae* infection is almost certainly higher than that reported by the Ministry. The normal incidence of *M. pneumoniae* is 0.6–3.1 per thousand per yr¹², yet an incidence of >300 per thousand per yr was seen among syndrome cases.

We have hypothesized that Spanish oil syndrome is a hyperacute form of *M. pneumoniae* infection in which the ingested oil acts as an adjuvant¹³. We draw an analogy to hyperacute experimental allergic encephalomyelitis (EAE) which is 100 times more active than ordinary EAE. Several lines of evidence support our hypothesis. First, many neurological and vascular symptoms of the syndrome have been reported as unusual complications of *M. pneumoniae* pneumonia^{14,16}. An oil adjuvant could have the effect of making these rare complications the norm, as in EAE. Second, the eosinophilia and perivascular cuffing associated with the syndrome are characteristic of chronic, allergic autoimmune diseases such as EAE. Third, a dual antigen aetiology would explain the observation that not all those people who ingested the oil became ill. The oil would be necessary but not sufficient alone to cause the disease. Finally, the ingestion of oil used in frying is not associated with the syndrome¹⁵. This observation argues against a free radical pathology as heat would create more free radicals. Alternatively, it is possible that some chemical constituent of the oil breaks down under heat to produce either nontoxic products or an inactive adjuvant. It is also possible that some of the oil was contaminated with *M. pneumoniae* introduced by unsanitary processing—an outbreak of pneumonia due to *M. pneumoniae* occurred just before the recognition of the syndrome².

Thus, we believe that our dual aetiology hypothesis deserves serious consideration as the most parsimonious explanation of all the available data.

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Jewel of the East

Colin Renfrew

THE great early civilization of the Indian sub-continent remained unknown until the year 1921, when Sir John Marshall made his remarkable — and quite unexpected — discoveries at Mohenjo Daro in the Indus Valley and at Harappa. With the recognition of Marshall's finds, knowledge of urban life in the area was taken back two thousand years before the days of Alexander the Great. At a stroke the Indus Valley, in what is today Pakistan and north-west India, was transformed in the eyes of scholars from something of a cultural backwater to one of the world's early heartlands of civilization.

Succeeding generations of scholars have done much to increase our knowledge, not only of the two great cities themselves, but of smaller towns such as Kalibangan in India and the coastal site of Lothal. The heyday of the Harappan civilization, around 2000 BC, is now very well documented: large areas of the cities have been cleared, and many aspects of urban life — notably the town planning reflected in the rectangular grid layout, and the very competent drainage systems — seem in advance of their contemporaries in Egypt or Mesopotamia. Only very recently, however, has the previous and formative period of the civilization been intensively studied. And only with the new data uncovered has it become possible to call into question the widespread assumption that the Harappan civilization was some sort of offshoot of Mesopotamian urbanism, a secondary phenomenon whose urban nature was largely due to outside influence.

New excavation at a number of sites, most notably Mehrgarh, has now taken the story very much further back, so that it is now possible to document settled farming life in the area before 5000 BC. And at Rahman Deri, the direct antecedents of the Harappan town there are taken back into the fourth millennium BC. The time is therefore ripe for a new work of synthesis to replace the earlier reviews of the civilization, amongst them Bridget and Raymond Allchin's *The Birth of Indian Civilisation* (Cambridge University Press, 1968).

With *The Rise of Civilization in India and Pakistan*, the Allchins bring their many years' experience of archaeology of those countries to bear on the wide range of material now available, from Afghanistan and Iran as well as the sub-continent itself. They offer an authoritative and well-judged survey which is likely to stand for many years as the most balanced treatment of this complicated subject.

The Rise of Civilization in India and Pakistan. By Bridget and Raymond Allchin. Pp.379. Hbk ISBN 0-521-24244-4; pbk ISBN 0-521-28550-X. (Cambridge University Press: 1982.) Hbk £25, \$49.50; pbk £8.95, \$14.95.

The authors begin with a thorough review of the prehistoric environments of the region, followed by two chapters which summarize the evidence from the Old Stone Age, and for hunter-gatherers and nomad pastoralists. The new evidence from the French excavations at Mehrgarh comes to the fore in the chapter on the first agricultural communities, and here the authors state their "belief that the indigenous developments of this region are of central significance for the subsequent development of Indian civilization in its widest sense". This view is substantiated in the following chapter on the Early Indus period, where several elements of continuity may be traced, from the preceding Neolithic through into the mature Indus civilization. To say this is not, however, to discount the effects of trade on the growth of early urbanism; relations between the Indus and Afghanistan and the Iranian plateau are now very well documented.

In succeeding chapters the authors deal with the collapse and aftermath of the Indus civilization, taking a properly cautious view about the supposed effects of hypothetical Indo-Aryan invaders at this time. In my own view there are arguments in favour of setting the advent of Indo-European speakers very much earlier. The supposed identification of a Dravidian (and therefore non-Indo-European) language in the Indus inscriptions rests more on supposition than on convincing argument.

During the succeeding Iron Age the Ganges seems to replace the Indus as the home of the more dynamic cultural developments. It seems paradoxical that we now understand rather more about the processes favouring the rise of the earlier Indus civilization than we do of that of classical India two millennia later.

The story is clearly told, with a systematic presentation of the archaeological evidence upon which it is based, and with a sound recognition of the limitations and gaps in that evidence. It was, presumably, an editorial decision to avoid all footnotes and text references, and to include only a "select general bibliography" at the back of the book, where further reading is suggested on a chapter-

by-chapter basis. This system certainly avoids disrupting the flow of the text, but scholars would certainly have preferred a more detailed reference system. The documentation of radiocarbon dates likewise seems too sketchy for systematic use. The authors' chronological judgement seems entirely sound, but even if individual dates are now too numerous to be listed in their entirety — which I doubt — it might have been possible to represent them all in one or more text figures, which would have illustrated the arguments more fully. The chronology, although doubtless secure in outline, is not yet fixed with any great precision, and the serious reader will wish to see more clearly the evidence upon which it is based.

These criticisms are worth making because *The Rise of Civilization in India and Pakistan* will justly acquire the status of a standard work: it is certainly the best treatment which we now have of the entire prehistory of the sub-continent. And it will surely stand for many years as the most convenient and reliable introduction, at a serious level, to this fascinating subject. □

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A ball of Cinderellas

M.J. Wells

Electrical Conduction and Behaviour in 'Simple' Invertebrates. Edited by G.A.B. Shelton. Pp.567. ISBN 0-19-857171-2. (Oxford University Press: 1982.) £40, \$98.

SCIENCE proceeds by a system of mutual backscratching. The "successful" neurophysiological preparations are those that attract sufficient disciples to form a caucus who will recommend further grants for further research in areas of mutual interest — *Aplysia*, locusts, squid giant fibres and a few other oddments from arthropods and molluscs, plus, of course, the obligatory rats and cats. Fair enough. It works. Having in effect agreed to limit the field to a few preparations, people at least talk the same language and that encourages them to speak to one another.

But in the long run lodes are worked out and real prospectors are needed, scouting around in unfashionable areas on the off-chance of the gold that will unleash another Klondyke. The odd thing about the authors of this collection of papers which specifically avoids both vertebrates and the

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annelid-mollusc-arthropod triad (practically the entirety of fashionable neurophysiology) is that the prospectors don't seem to be there for the money. Platyhelminthes and urochordates are not a way to a Nobel Prize. These people are in the outback because they like it. And with good reason; for most of this volume reports unfamiliar original research, which is somewhat more interesting than carrying out a series of exercises, adding dots and crosses to the i's and t's of an established literature.

Sadly, much of it is not all that exciting; it comes as no great surprise to know that most lower animals have nerves and propagate action potentials. But maybe we would not have predicted widespread epithelial conduction, the electrophysiological properties of the protozoan cuticle or some of the excitements to be dredged out of our own ancestors.

This is a source book. It summarizes a great deal of unfamiliar neurophysiological literature and it tells of the behaviour of animals — bryozoans,

ctenophores, sea anemones and jellyfish — that rarely feature in textbooks of ethology or comparative psychology. It is not, in itself, going to reform our views about nervous systems, though it may stimulate speculation about their evolution. The prospectors in these areas are still panning and if there are no very large nuggets to show for it as yet, the alert opportunist ignores the whole operation at his peril; something spectacular could turn up any day.

You may not want to spend £40 on a personal copy, but it might be wise to induce your departmental library to lay in Dr Shelton's book. No sensible social climber would have cared to back Cinderella's chances; but the ambitious neurophysiologist should be sufficiently aware of the suddenness of scientific change to cultivate at least a nodding acquaintance with the currently unfashionable. □

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Digging for defects in semiconductors

G.A. Baraff

Deep Levels in Semiconductors. By M. Jaros. Pp.302. ISBN 0-85274-516-8. (Adam Hilger/Heyden: 1982.) £24, \$53.

THE STUDY of deep-level point-defects is a part of the physics of semiconductors which, in turn, is a part of the physics of the solid state. Since the earliest days of solid state research, the physics of semiconductors has been an intellectually central and technologically essential part of the larger discipline. On the other hand, although the study of deep-level point-defects has been of technological importance, until recently it has been of only peripheral intellectual interest in the field of which it is a part.

That this situation is undergoing change is demonstrated by the number of recent conferences dealing, in one way or another, with the fundamental aspects of deep-level systems, by the number of sessions devoted to this topic in the biennial International Conference on Semiconductors, and in the number of recent review articles dealing with the subject. Milan Jaros's *Deep Levels in Semiconductors* is an expanded version of one such article.

Jaros is one of the few theorists who entered the field well before its present expansion, who is in part responsible for that expansion and who has remained an active contributor up to the present time. Indeed, his early work on the use of Green's functions to perform quantitative quantum mechanical calculations of deep-level wave functions undoubtedly motivated later workers to improve on the

beginnings he initiated. His book is primarily an account of these more modern developments. These include the use of self-consistent potentials in the Green's function formulation, the introduction of the *total* energy as the important concept in understanding the relationships between charge state and lattice distortion, and more attention to the evaluation of *observable* properties of deep-level systems.

The preface states that the book is intended for graduate students and researchers who are familiar with the basic facts of semiconductor physics. In my view the audience will turn out to be somewhat narrower than intended, because the strongest aspect of the book is in the philosophy and point of view expressed, while the weakest feature is the exposition of the mathematics of the underlying theory. To be able to follow the theoretical developments the reader should already have had exposure to the theory of defect systems, such as is provided by A.M. Stoneham's *Theory of Defects in Solids* (Oxford University Press, 1975), while to appreciate the philosophical flavour the reader should understand how completely dependent were the experimentalists of a decade ago on the insights provided by the effective mass theory of shallow defects. Jaros's early work stressed how seldom these insights remained valid for deep defects, and the present book continues to sound that warning.

In one sense, Jaros's book can be regarded as an updating of *Theory of*

Defects in Solids which is complete up until 1972. Jaros's first paper on deep levels was published in 1971, and his book is at its best on developments since 1976, the year in which his first Green's function-based calculation appeared. In style, however, it is quite different from Stoneham's treatise, being broad rather than both broad and deep. The reader who requires

the depth will nonetheless find the important original papers are prominently mentioned by Jaros, and will be better prepared to study them because of the overall perspective afforded by this book. □

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Plant ecology from a physiological viewpoint

Peter D. Moore

Physiological Plant Ecology I: Responses to the Physical Environment. Edited by O.L. Lange *et al.* Pp.625. ISBN 0-387-10763-0/3-540-10765-0. (Springer-Verlag: 1982.) DM 239, \$111.30.

THE NEW series of the *Encyclopedia of Plant Physiology* is to include four volumes devoted to physiological aspects of plant ecology. The objective of ecophysiology, according to the editors, is the explanation of processes in plant ecology in physiological terms. This has always been a concern of those plant physiologists with the vision to look beyond their own specialist spheres into the complex world of plant-plant and plant-environment interactions with which the field ecologist is concerned. It is now an expanding area of research in botany and these volumes aim to review its current state.

Part A (the first of the four volumes) deals with the reactions of plants to physical environmental factors; subsequent volumes will cover water relations and photosynthesis (Part B), the chemical environment and biotic interactions (Part C), with ecosystem studies, such as nutrient cycling and productivity, in Part D.

This first volume, with a few exceptions, is devoted to articles essentially dealing with light and temperature. The theme is introduced by an integrated account of the physics of radiation and the energy budgets of leaves and canopies, leading into a discussion of an essentially practical problem, that of defining and measuring photosynthetically active radiation. This is followed by a key chapter on the responses of plants to light intensity (or quantum flux density, to use the preferred term) by Olle Björkman, giving a lucid summary of the current state of sun-plant-shade-plant studies.

Responses to light which are not of a photosynthetic nature — seed germination, photomorphogenesis and photoperiodic phenomena — are becoming of increasing interest to ecologists and these processes are reviewed in some detail, together with the ecological implications of recent research, which are well presented and analysed. Potentially deleterious radiation, such as UV and ionizing radia-

tion, is then discussed, followed by a chapter on that peculiar habitat (from a radiant energy point of view), the aquatic environment.

The emphasis of the book then changes from radiant to thermal energy. Berry and Raison's general account of plant response to temperature uses the rather unusual approach of beginning with productivity and working through to sub-cellular responses; the reason for this may lie in their thesis that adaptation to thermal regimes in plants consists of the protection of their internal metabolic systems. More detailed chapters follow concerning sub-cellular responses, microbial populations and the ecological significance of both high and low temperature. These latter contributions consider both the problems of extreme conditions and those associated with seasonal or diurnal fluctuations.

Appended somewhat incongruously to the radiant theme of the volume are three topics, wind, fire and the soil. There are some connections between the fire and wind chapters and their predecessors in respect of temperatures and energy exchange, and the body of pertinent literature; together with their intrinsic ecological interest, this certainly justifies their inclusion in this position.

The final contribution on the soil environment, however, seems very out of place. It is brief (20 pages) and concentrates upon mechanical composition and structure and their influence on water movement. It would have been sensible either to devote this chapter to soil temperature or to hold it over to the next volume, which will include plant-water relations.

The reviews presented here are full and detailed and yet retain a readability which will make them invaluable as a source of reference for those approaching an ecophysiological topic from other disciplines. The book, and hopefully the series, will for many years continue to be the first resort of those seeking a reliable literature review in this sphere, even though the subject itself is developing so rapidly. □

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No end in sight

P.C.W. Davies

Infinity and the Mind: Science and Philosophy of the Infinite. By Rudy Rucker. Pp.338. ISBN 0-7108-0461-X. (Harvester/Birkhäuser: 1982.) £12.95, \$15.95.

ONE OF my earliest childhood memories is asking my father where outer space ends. "There is no end", he replied, then added cryptically, "If there was, what would lie beyond it?". I still remember that exquisite amalgam of awe, bafflement and disbelief at this first encounter with the infinite.

From time immemorial, man has been both scared and enchanted by infinity. Each culture has been forced to make its own peace with the idea. The Ancient Greeks revelled in its mystical significance, and the great classical philosophers grappled valiantly with paradox upon paradox. Those attributed to Zeno are best known. How, for example, can an arrow ever reach its target when it has to pass through an infinite number of places on its flight? The problem, for the classical philosophers, was their insistence that infinity can never actually be realized. It stands, like a forever unattainable goal, towards which processes can tend, but at which they can never arrive.

This classical tradition of *potential* infinity was only dispelled relatively recently, with the monumental work of the great nineteenth-century mathematicians, especially Georg Cantor. Cantor treated infinite sets as actual, completed, objects, and devised a collection of rules for manipulating infinite quantities. Thereafter, infinity became a respectable and rigorous mathematical concept, stripped of its paradoxical overtones.

It has not, however, lost its mystical appeal, and in this unusual book the author see-saws unpredictably between esoteric mathematical discourse and quasi-religious remarks. The early chapters introduce a number of circumstances where infinity is encountered in mathematics and physics. The discussion is reasonably elementary and straightforward. Later sections become much more advanced, and suddenly one finds oneself immersed in some pretty heavy technical analyses. Readers without a thorough mathematical grounding will get hopelessly out of their depth here, in spite of the liberal sprinkling of diagrams and the generous dimensions of the technical appendices.

One wonders, indeed, who the author is writing for. The intrinsic attraction of the subject, the author's fluent and convincing style, and his frequent allusion to the philosophical implications of the infinite, suggest that the book has a much wider potential readership than the technical level actually permits. Mystically-minded mathematicians and mathematically-minded mystics will feel very comfortable

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with it. Hard-nosed scientists or occult innumerates will not.

Rucker's "Absolute Infinity", variously identified with the self, the Universe, God, *The Absolute* and so on, emerges from a masterly discussion of the different degrees of infinity discovered by Cantor. One of the many surprises of the subject is that some infinities (e.g. the number of points in a continuous line) are bigger than others (e.g. the natural numbers 1,2,3, ...). Absolute Infinity is, if I interpret the author correctly, the grand-daddy of them all. This heady concept cannot, however, be grasped by rational thought at all but could, the author conjectures, be revealed mystically. The culmination of the book is an attempt, which I confess I found completely unconvincing, to identify zero, Zen-like, with Absolute Infinity. This is accompanied by a few tips to help the reader achieve the requisite state of consciousness whereby this apparently absurd conflation would become meaningful.

Another extraordinary claim follows a courageous and engaging plunge into Gödel's theorem and the paradoxes of self-reference. Expanding to tackle the wider issues of the soul, machine consciousness

and all that, the author abruptly announces "To exist is to have consciousness" (I am, therefore I think?), and goes on to confirm this astonishing position by asserting that the very page you are reading is conscious!

In summary, the author has written a remarkable account of a perennially fashionable topic in a peculiar mixture of styles. *Infinity and the Mind* is really two books in one, the first about the mathematician's infinity, the second about the mystic's infinity. The attempt to marry the two has obvious commercial value but, in my opinion, must be judged largely unsuccessful. Indeed, I am not sure that such a marriage is either desirable or feasible. Be this as it may, the book is a mine of mathematical information and a thoroughly stimulating read. I particularly enjoyed the section on the author's personal acquaintance with Gödel, and his dazzling account of bizarre orders of infinity which carry (apparently officially) quixotic names such as hyperinaccessible and indescribable. Everybody's father should read it. □

Paul Davies is Professor of Theoretical Physics at the University of Newcastle upon Tyne.

Separate the yolk from the white

D.M. Weir

Immunochemistry in Practice. By Alan Johnstone and Robin Thorpe. Pp.298. ISBN 0-632-00836-9. (Blackwell Scientific: 1982.) Pbk £9.80, \$19.95.

"The method is essentially [or a slight modification of] that described by Smith *et al.*, 1973".

How often do such statements appearing in the "Materials and Methods" section of a paper lead to a visit to the library, where one finds either that the journal cited is not purchased, is unavailable or, worse still, that Smith *et al.* may explain how to break the eggshell but refer back to earlier descriptions for the more technically demanding parts of the procedure? To step onto this treadmill is a sure way of dissipating a laboratory worker's energy and interest for the technique in question, perhaps even to raise doubts that the authors really want others to look closely at their methodology. And even when authors set out to give a full description of the procedure, the shorthand approach demanded by journal editors all too often ensures that the results are difficult to reproduce or even hazardous to perform without additional information and considerable laboratory skill.

Alan Johnstone and Robin Thorpe have set out to overcome these problems (including isolation of IgG from egg yolk) in their beautifully produced volume on immunochemical methods. For their resources they draw on their own

experience and are not too proud to take advice (suitably acknowledged) from workers with special experience in a particular technique.

There is enough theory in the paragraphs introducing each technique to ensure that the reader is not simply blindly following a recipe. The methods themselves are set out under three main headings: materials and equipment, procedure, and notes. Attention is drawn to toxic agents and carcinogens, and words such as "carefully" and "gently" are emphasized in italics. Sources of equipment and reagents are given and there is an appendix with names and addresses of suppliers, though there is often enough detail to enable the construction of equipment — an asset in these days of economic constraint.

The authors have made available to the large numbers of workers in immunology and related fields a wealth of invaluable information and guidance. In a book of under 300 pages it is not possible to provide full coverage of the subject, but a large number of widely used procedures are described using what the authors call "pertinent applications". Their efforts should go a long way to bridge the gulf that rapid technological developments in immunology can cause between immunologists and workers in other areas of biology. □

Donald M. Weir is Reader in Immunology at the University of Edinburgh Medical School.